

# Mango Breeding Support

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The Department of Agriculture, Fisheries and  
Forestry, Qld

Project Number: MG09003

## **MG09003**

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# **Mango Breeding Support**

## **Final Report**

**MG09003**

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Department of Agriculture, Fisheries and Forestry

AgriScience Queensland

Horticulture and Forestry Science

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This report details activities undertaken and results of the project "Mango Breeding Support" in which breeding, genomic and pathological technologies were researched and developed to improve the efficiency of mango breeding in Australia.

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# Contents

Media Summary .....	3
Technical summary .....	4
General Introduction .....	6
1. Technical Support for Increased Breeding Capacity and the Integration of Assisted Breeding Techniques .....	8
Introduction .....	8
Materials & Methods .....	8
Transfer of breeding program to Walkamin Research Station .....	8
Open pollination tree cages .....	8
Containerised paternal parent trees .....	10
Raising of pollinators .....	10
Progeny testing of open pollinated hybrids .....	11
Plant Material .....	11
Genomic DNA extraction .....	11
DNA amplification and Polyacrylamide Gel Electrophoresis (PAGE) .....	11
Data Analysis .....	11
Results & Discussion .....	12
Transfer of breeding program to Walkamin Research Station .....	12
Developing open pollinated tree cages .....	12
Growing Parental trees in large movable pots .....	12
Raising of pollinators .....	12
Open pollinated hybridisation .....	12
Progeny testing of open pollinated hybrids .....	13
2. Identify and test the application of gene markers for association with disease resistance and other traits .....	16
Introduction .....	17
Materials & Methods .....	17
Plant material .....	17
Phenotypic evaluation of mango fruit .....	17
Genomic DNA extraction .....	17
RNA extraction .....	18
EST library construction, sequencing and annotation .....	18
EST data mining .....	18
DNA amplification and capillary electrophoresis .....	18
Data Analysis .....	18
Results & Discussion .....	19
Analysis of mango EST-SSR sequences .....	19
Marker development and polymorphism of mango EST-SSRs within <i>M. indica</i> .....	19
Diversity analysis .....	20
3. Developing a recombinant linkage map .....	28
Introduction .....	28
Materials & Methods .....	28
Development of SNP markers .....	28
Plant material .....	28
Genomic DNA extraction .....	28
Results & Discussion .....	29
Future Plans .....	29
4. Screening for anthracnose resistance and developing a leaf assay for mango .....	30
Introduction and literature review .....	30
Materials & Methods .....	32

## Contents cont.

Test sample collection site .....	32
Pathogen isolate details .....	32
Culture storage and inoculum production .....	32
Resistance screening (Fruit) .....	32
Fruit collection and preparation .....	32
Artificial inoculation and assessment methods .....	33
2010/11 Season – Fruit screening - Experiment 1 .....	34
2010/11 Season – Fruit screening - Experiment 2 .....	34
2010/11 Season – Fruit screening - Experiment 3 .....	34
2011/12 Season .....	34
2011/12 Season – Fruit Screening - Experiment 1 .....	35
2011/12 Season – Fruit Screening - Experiment 2 .....	35
2012/13 Season .....	35
2012/13 Season – Fruit screening - Experiment 1 .....	35
2012/13 Season – Fruit screening - Experiment 2 .....	35
2012/13 Season – Fruit screening - Experiment 3 .....	35
Statistical analysis .....	35
Leaf bioassay for anthracnose tolerance.....	36
Leaf disc (laboratory method) .....	36
Detached leaf flush (laboratory method).....	36
Detached leaf flush (laboratory method) - Experiment 1 .....	36
Detached leaf flush (laboratory method) - Experiment 2 .....	36
Leaf flush (field method).....	37
Leaf flush (field method) - Experiment 1 .....	37
Leaf flush (field method) - Experiment 2 .....	37
Spray inoculated leaf flush (in field) method.....	37
Results.....	38
2010/11 Season - Fruit screening - Experiment 1 and 2 .....	38
2010/11 Season - Fruit screening - Experiment 3 .....	39
2011/12 Season - Fruit screening - Experiment 1 .....	39
2011/12 Season - Fruit screening - Experiment 2 .....	42
Season 2012/13 .....	42
2012/13 Season - Fruit Screening - Experiment 1, 2 and 3 .....	42
Leaf Bioassay for Anthracnose Tolerance .....	42
Leaf disc (laboratory method) .....	42
Detached leaf flush (laboratory method) .....	44
Leaf flush (field method).....	44
Discussion .....	45
Future activities .....	46
5. Develop test hybrid populations to use in genetic and genomic studies of disease resistance in mango. ....	46
Introduction .....	46
Materials & Methods .....	46
Results & Discussion .....	47
Technology Transfer .....	47
Seminar and conference and field day presentations.....	49
Visitors .....	50
Recommendations .....	51
Acknowledgments.....	52
Bibliography of Literature Cited.....	53

## Media Summary

The Mango Breeding Support project provided technical, research and development support to the Queensland-based, Australian Mango Breeding Program to develop and evaluate breeding systems and technologies that improve mango breeding efficiency. Adoption of efficient breeding support technologies will allow breeders to identify genes for desirable plant and fruit traits in parent varieties and incorporate those genes into new hybrid varieties more efficiently and rapidly.

The project compared traditional hand pollination methods with DNA marker assisted selection (MAS) open-pollinated methods to identify paternity and found both systems had advantages. Marker assisted paternity identification was not practical in all cases and relied on a greater range of technical skills and resources being available to the breeder. It is expected that MAS will become even more efficient when markers are available for production and quality traits in addition to parental identification.

Breeding for anthracnose resistance in mango is in its infancy. This project has identified several accessions in related *Mangifera* species with potential tolerance to postharvest anthracnose and tested the compatibility of these related species with the common mango and if the tolerance is transferable. The project investigated ways of determining a trees postharvest fruit disease resistance status in seedlings to avoid the up to 6 year wait for trees to crop.

Identification of genes and gene markers for plant development, stress response, fruit colour and flavour development was another goal of this project. Twenty five new expressed sequence tag (EST) derived single sequence repeat (SSR) DNA markers were identified and tested across a diverse range of germplasm. These markers were shown to be useful in determining genetic relationships, exploring potential pedigrees and estimating the genetic background of cultivated accessions of *M. indica*. They are the first reported EST-SSR markers suitable to cross-amplify in five wild *Mangifera* species.

The technologies that have been shown to be more efficient have been incorporated in to the Australian Mango Breeding Project. Other technologies being researched that are not yet fully developed to the stage where they can be adopted in a working breeding program are being progressed in other related research projects.

## Technical summary

Breeding mangoes is a long term endeavour, typically taking up to 20 years. There are several inherent biological obstacles to traditional breeding which contribute to the long breeding timeframes such as, a long juvenile phase, the polyembryonic nature of many cultivars, extensive natural fruit shedding and single seed per fruit. Mango has a short history of genetic studies and breeding and understanding of genetics is limited. If the Australian mango industry is to remain competitive with other fruits it is essential that continual gains in productivity and fruit quality are made. Improved genetics are an essential component of productivity and fruit quality gains. For mango breeders to improve mango genetics to keep pace with demands, the 20 year breeding time frame needs to be shortened and efficiency of breeding improved.

The objectives of this project were to support the core hybridisation program through developing breeding support technologies that improve breeding efficiency in the Queensland-based, Australian Mango Breeding Program. The science undertaken to improve breeding efficiency in this project fell under three areas, 1.) open-pollinated breeding systems, 2.) gene and marker discovery and 3.) discovering and measuring disease resistance.

The number of hybrids that the Australian Mango Breeding Program produces each year through hand pollination is limited by the time it takes to hand cross and mango flowering biology. Open-pollination breeding systems can generate larger numbers of hybrids; however the random nature of open-pollination produces many non targeted hybrids that need to be grown-out to maturity before culling, at great expense. If open-pollination is coupled with effective marker assisted selection (MAS), culling can occur before field planting. However, the costs and efforts of an open-pollinated MAS system may outweigh the benefits. This project developed and tested the practical application of such a system in mango.

The project concluded that open-pollination, linked with expressed sequence tag derived single sequence repeat (EST-SSR) markers for paternity testing was not practical in all families, however, in appropriate families it was a useful supplement to hand pollination methods. With the future development of multiple gene based single nucleotide polymorphism (SNP) markers the application will widen and the cost/benefit balance may well change in favour of open pollination systems.

Expressed sequence tag (EST) sequences generated in an earlier project were selected for their placement in putative genes of interest in plant development, stress response, fruit ripening and colour and flavour development. The selected ESTs were mined for SSRs and polymerase chain reaction (PCR) primers were developed. Twenty five EST based SSR markers were identified and analysed for their allele sizes and compared across a diverse group of 32 genotypes of *Mangifera*. Allele data was used to generate a diversity dendrogram for the 32 genotypes that showed a high level of diversity. When the 25 new EST-SSRs were combined with 11 previous known SSRs, the level of diversity detectable was significantly increased. No correlations were found between the new EST-SSRs and phenotypic data for background skin, blush and pulp colours of fruit.

These 25 new EST-SSR markers have increased the number of informative microsatellite markers available for *Mangifera* species, in determining genetic relationships, exploring potential pedigrees and estimating the genetic background of cultivated accessions of *M. indica*. They are the first reported EST-SSR markers suitable to cross-amplify in five wild *Mangifera* species.

A recombinant linkage map has been developed in collaboration with the USDA that will combine Australian and American mango sequence data. Initially, 144 Australian SNP markers were



analysed across the hybrid family 'Irwin' x 'Kensington Pride' where over 20 "linkage" groups were found. To improve the genetic linkage map further SNPs developed by the USA will be combined with the Australian SNPs already screened. These will be run across all the hybrid families and the resulting map reported in the follow-on HAL project 'Integrating genomics into an applied breeding program' (MG13002). This map and Australian phenotypic data will form the basis for quantitative trait loci (QTLs) identification.

Incorporation of tolerance to postharvest anthracnose into new hybrid varieties has the potential to reduce pre- and postharvest chemical usage and to increase the shelf life of mango. This project has been screening genebank accessions for tolerance to anthracnose, developed a leaf assay for anthracnose tolerance and developing a hybrid population segregating for anthracnose tolerance. More than 105 mango accessions have been tested in this and previous projects through natural and artificial inoculation methods. One accession *Mangifera laurina* cv. Lombok has stood out with significant tolerance. This accession has been hybridised with advanced breeding lines generating over 65 progeny to date. These progeny are immature, so anthracnose tolerance segregation patterns are not yet determined. Much work has been done on developing a leaf assay for anthracnose tolerance for early identification of a tree's tolerance status. Development of the leaf assay has not been completed in this project and will continue in other projects.

## General Introduction

Since the 1950's researchers and industry have been involved in genetic improvement and breeding of Australian mangos. This research has taken several approaches such as varietal introduction and evaluation (Beal 1981; Johnson and Robinson 1997; Scholefield et al. 1984; Shepherd 1972; Winston 1984; Winston 1993; Wright and Bally 1984a), selection of open pollinated monoembryonic seedlings (Bally 1998; Wright and Bally 1984b), clonal selection within the 'Kensington Pride' population (Bally 1995; Bally et al. 1996; Bally et al. 2002; Johnson 1995; Johnson and Robinson 1997; Winston 1984), assisted open pollination (Whiley 2000; Whiley et al. 1993; Whiley and Saranah 1995) and controlled closed pollination (Bally 2002; Kulkarni et al. 2002). The relative advantages and disadvantages of these approaches were reviewed by Bally (2006). Each of these approaches has contributed to the availability of genetic material available for industry and a better understanding of mango genetics. However these approaches must keep pace with the changing production, marketing and consumer demands of mango cultivars and the need for continual improvement to provide the best and most competitive genetics as a base for the Queensland and wider Australian mango industries in the future.

Between 1994 and 2004 four research agencies (CSIRO, NT DPI&F, Ag WA and QDPI&F) collaborated in the National Mango Breeding Program (NMBP) to develop new mango hybrids through hand pollination techniques (Bally et al. 1999). Three new hybrid cultivars originating in this program are in the process of being released to commercial partners. Although the hand pollination approach used by the NMBP was technically advanced, its efficiency is low compared with some other crops because the inherent reproductive, pollination and production biology of mangoes. Only 0.1 to 8% of flower panicles crossed produce hybrid fruit.

After 2004 when CSIRO and the NT ceased involvement in mango breeding, Queensland continued the Australian Mango Breeding Program (AMBP) with support from Ag WA in some activities. The Queensland Department of Agriculture Fisheries and Forestry has also recognised the low efficiency of current mango breeding technologies and supported the mango fruit genomics initiative (MFGI) (Clarity project number 2368) to lay down the groundwork for the development of molecular tools to improve the efficiency of mango breeding and improve the understanding of mango genetics.

Molecular markers are a versatile tool for a large number of applications in genome analysis, ranging from localization of a gene to improving plant varieties by marker assisted selection. The application of these markers can reduce the timeframe of developing new and better varieties for commercialisation. They have also become extremely useful for phylogenetic analysis adding new dimensions to mango evolutionary development (Dillon et al. 2013, Dillon 2009; Honsho et al. 2005; Schnell et al. 2006; Viruel et al. 2005).

The disease anthracnose caused by the fungal organism *Colletotrichum gloeosporioides* Penz. is a major problem in a range of horticultural industries globally (Jefferies et al. 1990) and mango (*Mangifera indica*) is no exception.

In Australia, anthracnose is managed by an integrated programme (Lim and Khoo 1985) consisting of hygiene practices (pruning of dead material from the tree canopy), regular fungicide applications (protectant and systemic), a balanced nutrient programme and the use of post-harvest treatments such as hot water on its own or a combination of hot water and fungicide dips (Muirhead 1976; Dodd et al. 1991). However, even if the above treatments are carried out to a rigorous schedule, the programme is still not 100% effective at managing the disease. High

quality genetic tolerance or resistance to anthracnose would provide an additional strategy for managing the disease and reduce pressure on chemical control.

Previous investigations by Grice et al. (2008), Akem et al. (2007) and Grice and Bally (2007) at Ayr and Mareeba identified an accession of *Mangifera sp.* with significant tolerance to postharvest fruit anthracnose and a hybridisation program to incorporate this tolerance was initiated. To efficiently incorporate anthracnose tolerance into new cultivars a system of rapidly testing parents and progeny was needed as trees can take up to 6 years to bear fruit.

The objectives of this project were to support the Australian Mango Breeding Program to improve breeding efficiency in mango by developing and boosting technical and scientific capacities that have potential to shorten the time frame from cross pollination to cultivar release. The project supported and investigated improved breeding efficiency in the following areas:

- Technical Support for Increased Breeding Capacity and the development and integration of an open-pollination breeding system with marker assisted breeding techniques.
- Identifying and testing gene makers for breeding traits and/or their controlling mechanisms.
- Developing a recombinant linkage map as a tool in the pathway to discovering quantitative trait loci (QTLs).
- Continuing to screen the Australian mango genebank for resistance/ tolerance to anthracnose.
- Developing a leaf assay for mango anthracnose resistance.
- Continuing the developing a hybrid population segregating for disease resistance.

The development of these technologies and their application in the Australian Mango Breeding Program will improve breeding efficiency by enabling genes for specific traits to be identified and incorporated into new hybrid mango varieties quicker than in the past, which in turn will provide the Australian mango industry with cultivars that have competitive advantages in productivity and fruit quality.

# **1. Technical Support for Increased Breeding Capacity and the Integration of Assisted Breeding Techniques**

Component Personnel: Dr Ian Bally, Dr Natalie Dillon and Ms Cheryl Maddox

## **Introduction**

This activity of the project has supported tree management and maintenance costs of over 300 additional trees associated with these project activities and 0.75 FTE employment of Ms. Cheryl Maddox who undertook most of the day to day technical activities of the project including:

- Coordinating farm, technical and scientific staff on the movement of the breeding program from Southedge Research Station (SRS) to Walkamin Research Station (WRS) due to the sale of Southedge research station in early 2013.
- Developing the day to day methodologies for open pollinated breeding.
- Developing methods to efficiently raise and introduce pollinating insects to the cages to carry out the open pollination hybridisation.
- Growing parental trees in large movable pots.

Hand pollination breeding techniques are used by only a few mango breeding programs, mainly India and Australia. Other mango breeding programs use assisted open pollination methods with caged parental trees in close proximity and allow insects to pollinate the flowers. These open pollination methods use less labour in the pollination process and generated larger numbers of hybrids compared to hand pollination techniques. Despite the increased production and labour saving with open pollination methods, additional resources are needed to maintain larger numbers of progeny in the field and for molecular identification of progeny and markers.

This project developed and tested an open pollination breeding process in parallel with hand pollination methods to develop practical efficient methods of open pollination and associated marker assisted selection. Progeny of four test open pollination families were subjected to MAS to identify their paternal parentage using microsatellite markers and polyacrylamide gel electrophoresis (PAGE). This process allows the breeders to accurately identify both parents of a hybrid progeny and make appropriate selection and culling decisions.

## **Materials & Methods**

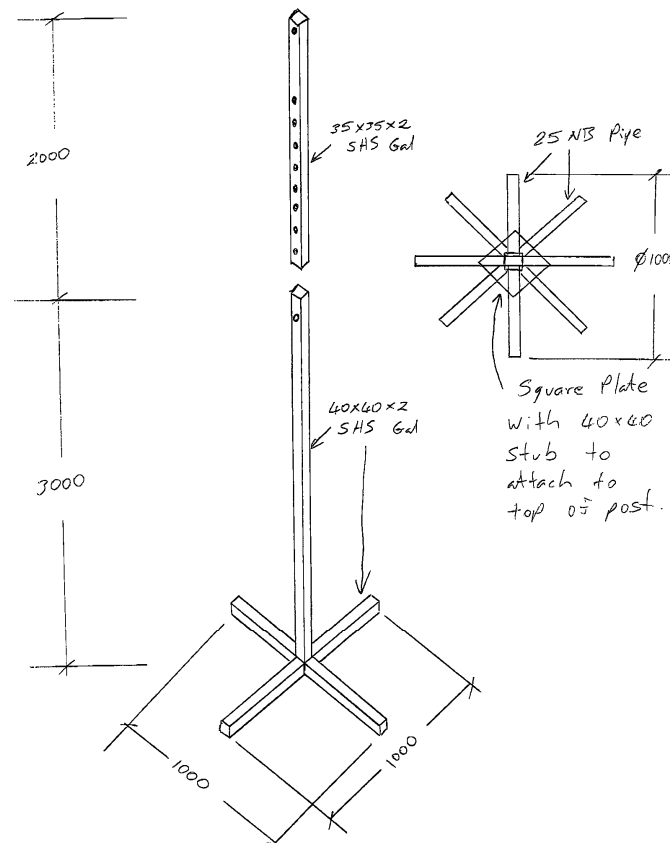
### ***Transfer of breeding program to Walkamin Research Station***

The sale of Southedge Research Station forced the transfer of the whole mango breeding program and mango genebank to Walkamin. This involved propagating the entire mango genebank, establishing a block of parental varieties on Walkamin for future hybridisation, moving all elite selections identified in earlier breeding and establishing blocks to accommodate any new hybrid progeny produced.

### ***Open pollination tree cages***

The development of methodologies for open pollinated breeding was undertaken over the 2010, 2011 and 2012 flowering seasons and consisted of constructing insect proof nets over sets of parental trees and introducing pollinator insects to the nets to facilitate the technique.

Several net support mechanisms structures were tested for their suitability to be rapidly erected, fit a range of tree sizes and allow efficient pesticide spraying of the trees within the nets. The best structure consisted of adjustable height central supports made from box steel with a four pronged base and a six pronged roof support (Figure 1). Height adjustment was achieved by the central pole having an outer box steel pole attached to the base and an inner box steel pole attached to the top and a bolt through both inner and outer box sections to secure the required height. The cage frames were constructed from 50 mm high density polyethylene pipe connecting the central support top prongs to star pickets outside the periphery of the trees being caged (Figure 2). The insect proof netting used was a translucent Raschel wrap knitted, HDPE



**Figure 1. Central frame support for net tree cages**



**Figure 2. Insect proof netted tree cages for assisted open pollination breeding. A) is an early model with insufficient support. B) is improved model with central steel support.**

monofilament, fruit fly netting with a hole size of approximately 2 to 3 mm, manufactured by NetPro. Access to the trees inside the nets was through an overlapping section of net that was parted for access but hangs without an opening when not being entered. Cages were constructed over the crossing parent trees after pruning to restrict extra high or spreading trees.

### ***Containerised paternal parent trees***

To facilitate multiple combinations of breeding parents being used in the open pollinated cage system, 20 paternal (pollen donor) parent trees were grown in transportable 150 litre pots. Each pot was placed on a small galvanised steel pallet to facilitate their removal by tractor (Figure 3). These transportable containerised trees were available to be moved into open pollination cages (Figure 4) for the duration of the flowering season to facilitate cross combinations where the desired parent trees were not growing adjacently in the field.



**Figure 3. Transportable containerised parent trees ready to be moved into netted tree cages during the flowering season. .**



**Figure 4. Containerised pollen parent tree installed inside netted cage to act as a pollen donor in the open pollination process.**

### ***Raising of pollinators***

Prior to panicle emergence pollinating flies were raised and introduced into the netted enclosures to facilitate pollination between the enclosed trees. Flies were raised by attracting wild flies to lay eggs in decaying canned fish or pet food. After the eggs had hatched and the maggots had begun to feed they were enclosed in small cages with a base tray covered with a mix of peat and vermiculite in which they pupated. The trays containing pupating larvae were transferred to the netted tree cages where the pupae hatched and the flies were able to move freely in the caged enclosures (Figure 5 and Figure 6) to pollinate the enclosed flowering trees.



**Figure 5.**  
Pollinator flies on  
leaf inside cage



**Figure 6. Pollinator flies on the inside  
of the net cage.**

### ***Progeny testing of open pollinated hybrids***

#### ***Plant Material***

Three hundred and thirty six hybrid mango progeny from four open pollinated families were harvested for testing in 2010. The parental combinations were:

1. Tommy Atkins♀ x NMBP 1243♂
2. NMBP 1243♀ x Tommy Atkins♂
3. MNBP 1201♀ x NMBP 1243♂
4. NMBP 1201♀ x NMBP 4069♂

Seed was recovered from the harvested fruit of the female monoembryonic parents of each test family, germinated and planted into 5 litre pots housed under 50 % shade.

#### ***Genomic DNA extraction***

Leaf tissue was collected from each of the hybrid progeny and the four parent trees for genomic DNA extraction. Extractions were performed using DNeasy® 96 Plant extraction kits (Qiagen, Germany) according to the manufacturer's instructions. Extracted DNA was stored at 4°C and diluted to a final concentration of 10 ng/μl prior to PCR amplification.

#### ***DNA amplification and Polyacrylamide Gel Electrophoresis (PAGE)***

SSR polymerase chain reaction (PCR) amplifications were carried out in a T100™ Thermal Cycler (BioRad). The amplifications were conducted in a total of 20 μl containing 1x *Taq* Buffer containing (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (Thermo Scientific; formerly Fermentas; Victoria, Australia) 2 mM MgCl<sub>2</sub>, 0.25 mM dNTPs, 0.3 μM of each primer (LMMA10) and 1.0 unit *Taq* DNA polymerase (Thermo Scientific). Thermal cycling conditions included an initial denaturation at 94°C for 3 min followed by 10 cycles of 30 s at 94°C, 1 min at 50°C (-1°C / cycle), and 90 s at 72°C with a further 30 cycles of 30 s at 94°C, 1 min at 40°C, and 90 s at 72°C with 10 min at 72°C for a final extension. PCR amplicons were separated by 12% PAGE; 10 μl of PCR product was mixed with 2.5 μl of EZ-Vision prior to a 120 min separation at 100 V.

#### ***Data Analysis***

Allele data analysis was performed manually for fragment size determination and for allelic designations (discrimination).



## Results & Discussion

### ***Transfer of breeding program to Walkamin Research Station***

The transfer of the mango germplasm from SRS to WRS is almost complete with only a few individual trees still to be regrafted and planted at WRS. The Maud Creek block on WRS now houses the Australian Mango Genebank (335 accessions), a 130 tree breeding crossing block with parents for future hybridisation and a 1,978 hybrid breeding progeny block (Figure 7).



**Figure 7. Hybrid progeny planted on Walkamin Research Station**

### ***Developing open pollinated tree cages***

The cage system worked well trapping introduced pollinating insects and excluding unwanted predatory insects such as fruit spotting bugs.

### ***Growing parental trees in large movable pots.***

The main drawback with containerised parental trees is the timing of flowering. Flowering in the potted trees often is not in synchronisation with the field grown parent due to different growing and stress conditions of trees in pots. Flower induction technologies used by commercial growers in other countries may help in this situation. However these techniques were not tested in this project.

### ***Raising of pollinators***

Raising pollinators was relatively straight forward however, the time period needed to raise the flies varied greatly between seasons due to changes in ambient temperature. At least a month lead time to collect, grow and hatch larvae for pollination is needed during the cool winter months.

### ***Open pollinated hybridisation***

The open pollination system of generating hybrids in insect proof cages that enclose trees growing in the ground or in containers is still under evaluation. The cage system works well in enclosing introduced pollinating insects and excluding unwanted predatory insects such as fruit spotting bugs. However, there are some drawbacks with generating hybrids using the open pollinated cage system. These include the need to have synchronised flowering between both parents. This has not always been the case over the period of this project. Often caged trees did not flower or there was no overlap with flower timing of the potted parent trees enclosed in the nets. Although open pollinated hybridisation systems avoid the expensive process of hand pollination, the system is expensive and time consuming in other areas. Open pollination systems require a significant labour input throughout the year for activities such as preparing, maintaining and moving potted



parent trees, erecting cage nets over trees, collection and germination of large numbers of potential hybrid seeds, collecting and extracting DNA from progeny tissue samples and molecular marker analyses.

In breeding programs where staff labour is limited, open pollination methods may not provide the savings and efficiencies they can in larger programs. However, in some situations where parental trees are planted next to each other in the field efficiencies may be improved but this requires the parental trees to be identified and planted together 4 to 6 years before crossing. The newly established crossing block at Walkamin Research Station has parents planted together to facilitate open pollination in the future. Where parents are not in close proximity, hand pollination can take advantage of female and male trees located remotely from each other and relatively low numbers of pollen parent flowers are required for successful pollination.

The successful development and application of molecular markers to determine paternity in open pollinated breeding populations has been a significant advance in mango breeding methods. The efficiencies gained through the application of molecular markers will vary depending on tree positions, pedigrees, compatibility of parents and the timely application of molecular technologies. These factors will change from one season to another with flowering patterns of parental trees. We see this technology as a valuable addition to the hand pollination methods and expect they will be used in parallel with hand pollination in situations where floral synchrony and compatibility occur.

The selection rates of progeny based on molecular markers for paternity will depend on the pedigree and compatibility of the parents and other pollen donors in the vicinity of the cross. There may be some parental combinations that are more suited to this technology than others. Identification of suitable parental combinations for molecular paternity testing has not been done for many families and currently relies on trial and error. Future research into mango compatibility rates will assist in determining the most appropriate breeding methodologies to use for any given family.

Determining parentage using molecular markers visualised with PAGE gel systems has been shown to be useful, however the current use of SSR markers visualised with PAGE gel systems is slow and expensive and does not always provide the answers with closely related parents. In the future SSR markers are likely to be replaced by SNP markers that are more abundant than SSRs and so will be able to distinguish between related individuals more accurately and will be cheaper to run.

### ***Progeny testing of open pollinated hybrids***

Of the 336 hybrid progeny sampled for testing, 61 or 18% failed to provide sufficient DNA for analyses. The mango leaf tissue used in this experiment were older leaves that are more difficult to grind in the narrow tubes provided with the DNA extraction kit. This led to many of the samples grinding poorly and subsequently resulted in a lower efficiency of genomic DNA extraction and poorer quality DNA.

The hybrid family 'Tommy Atkins' x 'NMBP 1243' (an 'Irwin' x 'Kensington Pride' hybrid) had 54 hybrid leaf samples collected. Of these 54 samples, 10 failed PCR amplification and two individuals gave an identical genotype to that of the male parent ('NMBP 1243') genotype. This gave a total of 42 putative hybrid progeny for this family (78%).

The hybrid family 'NMBP1243' x 'Tommy Atkins' had a total of 76 hybrid leaf samples collected. Of these 76, nine failed PCR amplification and 45 individuals gave an identical genotype to that of

the male parent ('Tommy Atkins'). This gave a total of 22 putative hybrid progeny for this family (29%).

The hybrid family 'NMBP 1201' (an 'Irwin' x 'Kensington Pride' hybrid) x 'NMBP 1243' had a total of 48 hybrid leaf samples collected. Of these 48, seven failed PCR amplification with 15 individuals giving an identical genotype to that of the female parent ('NMBP 1201') and seven giving an identical genotype to that of the male parent ('NMBP 1243'). This gave a total of 19 putative hybrid progeny for this family (40%).

The hybrid family 'NMBP 1201' x 'NMBP 4069' had a total of 158 hybrid leaf samples collected. Of these 158, 35 failed PCR amplification, and two individuals had the similar genotype pattern of 'NMBP 1243'. Fifty one individuals gave an identical genotype to that of the male parent 'NMBP 4069' while no individuals had a genotype of the female parent 'NMBP 1201'. This gave a total of 68 hybrid progeny for this family (43%).

The process of using one molecular marker (LMMA10) to determine parentage of open pollinated hybrid mango seedlings from the field was successful in identifying those seedlings with similar genotypes to one of the two parents. Where the genotype is identical to that of the female parent it can be considered as a self pollinated hybrid. This marker can then be useful as a quick and cheap method for confirmation of self pollinated hybrids. However, where the genotype is identical to that of the male parent the genetics become more difficult to interpret. Possible scenarios include human error in collection of samples or processing, or failure of the microsatellite marker due to genetic variation within the progeny. This is where the use of multiple microsatellite markers is beneficial to overcome these issues of genetic variation with allele data from multiple markers to assist with resolving these issues. It was more difficult to confirm the paternity of hybrid mango progeny from the netted open pollinated families. In a breeding program where selected parents of open pollinated families often have similar or common pedigrees it is not always possible to get unique alleles that are large enough to be separated on a PAGE gel system. When this is the case paternity testing is difficult.

A range of allele sizes for hybrid genotypes were identified, by comparison of bands on PAGE with one standard marker, within each of the hybrid families indicating the possibility that pollen from an outside parent may be the paternal parent to some progeny. Use of further molecular markers to identify the outside parent would increase the accuracy of the PAGE analysis. However every additional marker significantly increases the work load and the cost of the analysis. As the numbers of putative hybrid individuals has been decreased by 55% with the use of one microsatellite marker it may then be cost-effective to run these remaining samples with more accurate fluorescent-based markers via capillary electrophoresis to confirm parentage. In the future the development of single nucleotide polymorphic (SNP) markers for mango will be cheaper per data point to run than microsatellite markers. Multiple SNPs that can be run simultaneously are of greater value than microsatellite markers as they have the potential to be linked to phenotype.

The selection rates of progeny based on molecular markers for paternity will depend on the pedigree, compatibility of the parents and other pollen donors in the vicinity of the cross. These factors will change from one season to another with flowering patterns of parental trees. There may be some parental combinations that are more suited to this technology than others. Identification of suitable parental combinations for molecular paternity testing has not been done for many families and currently relies of trial and error. Future research into mango compatibility rates may assist in determining the most appropriate breeding methodologies to use.

Determining parentage using SSR markers visualised with PAGE systems is possible however, the process is slow and expensive and does not always provide the answers with closely related parents. In the future SSR technology is likely to be replaced by SNP markers that will be more abundant than SSRs, be able to distinguish between related individuals more accurately and cheaper to run.

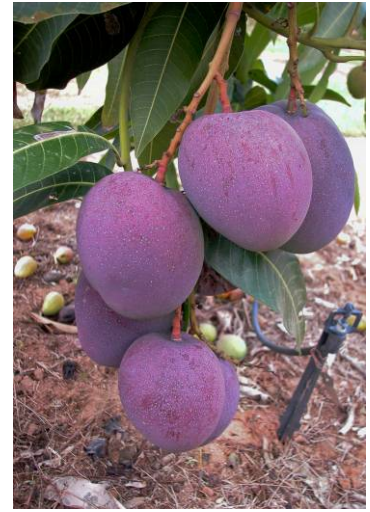
We do not see the open pollination method replacing the hand pollination method in mango breeding in Australia. However it will be a valuable alternative method to be used in parallel with hand pollination in situations where floral synchrony and compatibility occur.

**Table 1. Parental combinations for production of hybrid progeny for parentage analysis.**

Family		No. hybrid progeny	No. failed PCR	No. female genotype (self pollinated)	No. male genotype	No. putative hybrids (%)
Female parent	Male parent					
Tommy Atkins	1243	54	10	0	2	42 (78%)
1243	Tommy Atkins	76	9	0	45	22 (29%)
1201	1243	48	7	15	7	19 (40%)
1201	4069	158	35	0	51	68 (43%)
		<b>336</b>	<b>61</b>	<b>15</b>	<b>105</b>	<b>151 (45%)</b>



NMBP 1243 X Tommy Atkins



NMBP 1201 X NMBP 1243



NMBP 1201 X NMBP 4069



**Figure 8. Mango cultivars used as parents in open pollinated and marker assisted selection**

## 2 Identify and test the application of gene markers for association with disease resistance and other traits.

Component Personnel: Dr. Natalie Dillon

### Introduction

Several activities were undertaken to identify and test the application of gene markers for the mango breeding program.

The work expanded on the research undertaken in an earlier project "Mango Fruit Genomics Initiative" (MFGI) where a total of 24,840 expressed sequence tag (EST) sequences were generated from five *M. indica* cDNA libraries prepared from 'Kensington Pride' red leaf, fruit, flower and root and 'Irwin' red leaf. Using strict threshold criteria, 1,802 SSRs were identified within the 24,840 EST sequences (7%). With closer inspection of the SSR and SNP marker data there were fewer sequences associated with stress/defence response genes than had originally been expected. However, a total of eight EST-SSR markers associated with stress/defence response genes were included in the selection of 25 EST-SSRs developed for marker screening. The other EST-SSR markers developed were within genes associated with plant development, colour and flavour development pathways, traits of importance to mango breeders and where previously measured phenotypic data was available within a selection of genetically diverse *Mangifera* accessions.

### Materials & Methods

#### *Plant material*

Thirty-two mango and other *Mangifera* species accessions maintained at the ANMG at Southedge Research Station, Mareeba (16°45'S, 145°16'E), and at Ayr Research Station (19°31'S, 147°22'E), Queensland, Australia, were used in this study (Table 2). All accessions were grafted onto the uniform polyembryonic rootstock of the cultivar 'Kensington Pride'. EST libraries were constructed from 'Kensington Pride' red leaves, flowers, fruit pulp and skin, and roots and 'Irwin' red leaves. 'Kensington Pride' was selected as it is the predominant variety grown in Australia. 'Irwin' was selected for its high fruit colour, high productivity, semi-dwarf characteristics.

#### *Phenotypic evaluation of mango fruit*

Pulp colour, background skin colour and blush colour were evaluated on the majority of the accessions used. At harvest, 10 fruit from each accession (Table 2) were sampled evenly from all quadrants of each tree. Fruits were transported to the laboratory within two hours of harvest, where they were dipped in 1 ml L<sup>-1</sup> of the fungicide carbendazim at 52°C for 5 min and subsequently held between 22°C and 24°C to ripen. All colour evaluations were undertaken on fruit at the eating ripe stage. Colour was evaluated categorically with the scales listed in Table 4 and electronically using the Hunter *L. a. b.* scale (Hunter 1948).

#### *Genomic DNA extraction*

Where possible, young fresh flushing leaf buds were collected for genomic DNA extraction. Where leaf buds were not available, the youngest leaf material available at the time was collected. DNA extractions were performed using DNeasy® Plant extraction kits (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration and integrity was checked by analytical 1% (w/v) agarose/TBE gel electrophoresis. Extracted DNA was stored at 4°C and diluted to a final concentration of 10 ng/μl prior to PCR amplification.

### **RNA extraction**

RNA was extracted from 'Kensington Pride' red leaf, fruit skin, fruit flesh, flower and root tissues, and from 'Irwin' red leaf tissue using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, Sydney, Australia) according to the manufacturer's instructions.

### **EST library construction, sequencing and annotation**

*(This section of work was undertaken within the Mango Fruit Genomics Initiative supported by Agri-Science Queensland, a division of the former Department of Employment, Economic Development and Innovation. It was not a part of the HAL-funded Mango Breeding Support project.)*

Extracted RNA was used to prepare cDNA libraries using the SuperScript Plasmid System for cDNA Synthesis and Cloning (Invitrogen) as per the manufacturer's protocol. Single pass, 5'end sequencing was performed at the Australian Genome Research Facility (AGRF) using Applied Biosystems 3730xl capillary sequencers. The raw chromatogram files were quality clipped using phred (Ewing and Green 1998; Ewing, Hillier et al. 1998) and vector sequences were removed using CrossMatch within the Staden package (Staden et al. 2000). The Staden output files were parsed using Perl scripts prior to assembly using cap3 (Huang and Madan 1999). Putative functions of resulting contig and singleton sequences were assigned on the basis of similarity to *Arabidopsis thaliana* peptide sequences (TAIR8; Swarbreck et al. 2008) using BLASTx (Altschul et al. 1997). Bioinformatics analysis was performed at the Queensland Facility for Advanced Bioinformatics (QFAB).

### **EST data mining**

EST sequences were mined for SSRs using Perl scripts with thresholds of six repeat units for dinucleotide repeats and four repeat units for tri-, tetra-, penta-, and hexa-nucleotide repeat motifs. Sequences with putative SSRs were passed to primer3 (Rozen and Skaletsky 2000) and PCR primers were designed where sequence context permitted.

A set of 25 EST-SSRs were further analysed (Table 3). They were selected based on their placement within putative genes of interest in plant development, stress response, and fruit ripening and colour development. Primer pairs were synthesised by Applied Biosystems (Foster City, CA) and forward primers were labelled at the 5'end with fluorescent dyes 6FAM, VIC, PET or NED.

### **DNA amplification and capillary electrophoresis**

EST-SSR polymerase chain reaction (PCR) amplifications were carried out in a Veriti® Thermal Cycler (Applied Biosystems). The amplifications were conducted in a total of 6 µl containing 1x ImmoBuffer (Bioline Pty Ltd, Alexandria, NSW, Australia) 1.5 mM MgCl<sub>2</sub>, 1.25 mM dNTPs, 0.33 µM of each primer and 0.2 units Immolase™ DNA polymerase (Bioline). Thermal cycling conditions included an initial denaturation at 95°C for 15 min followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, and 60 s at 72°C with 10 min at 72°C for a final extension.

PCR amplicons were separated by capillary electrophoresis on a 3730 DNA Analyzer (Applied Biosystems); 1 µl of PCR product was mixed with 10.4 µl of HiDi formamide and 0.06 µl of the size standard LIZ 500 (Applied Biosystems) prior to a 60 min separation at 230 V, 32 amp.

### **Data Analysis**

Allele data analysis was performed using the GeneMapper software version 3.7 (Applied Biosystems) for internal standard and fragment size determination and for allelic designations. Automated allele calling was performed initially and flagged data then called manually.

The genetic similarities between the genotypes were calculated using Cavalli-Sforza distance (Cavalli-Sforza and Edwards 1967). Dendrograms were constructed using the neighbour-joining (NJ) method (Saitou and Nei 1987) and rooted on the mid-point. The robustness of the dendrograms was assessed by creating 1000 bootstrap replicates of the data and then generating a majority rule consensus tree. Distance calculations, tree construction and bootstrapping were all performed in PowerMarker V3.0 (Liu and Muse 2005). Polymorphism information content (PIC) values for diversity analysis were calculated for each locus according to the formula:  $PIC = 1 - \sum P_i^2$ , where  $P_i$  is the frequency of the  $i$ th allele in examined genotypes (Liu 1998). EST-SSR and phenotypic data (background skin colour, blush colour, pulp colour of fruit (Table 2) were evaluated by estimating cophenetic correlation using Mantel's matrix correspondence test with 10,000 permutations (Mantel 1967). The Cavalli-Sforza distance was used to form the distance matrices for the EST-SSR marker data and the Euclidean distance or simple-matching distance was used for the phenotypic data.

## Results & Discussion

### **Analysis of mango EST-SSR sequences**

A total of 24,840 EST sequences were generated from five *M. indica* cDNA libraries prepared from 'Kensington Pride' red leaf, fruit, flower and root and 'Irwin' red leaf. BLASTx analysis of the quality clipped and trimmed ESTs identified 22,726 sequences (93%) with matches against *A. thaliana* amino acid sequences at  $e$  values less than  $1 \times 10^{-10}$ . These libraries generated approximately  $14.5 \times 10^6$  nucleotides of mango sequence with the average length of EST sequences being 578 nucleotides. Using the threshold criteria as outlined in the materials and methods, 1,802 SSRs were identified within the 24,840 EST sequences (7%). 'Kensington Pride' red leaf ( $n = 454$ ) and root ( $n = 438$ ) cDNA libraries showed the highest number of EST-SSR sequences. The lowest number of EST-SSR sequences were identified in 'Irwin' red leaf ( $n = 286$ ) and 'Kensington Pride' fruit skin and flesh ( $n = 296$ ) cDNA libraries. A single SSR each was present in 866 unigenes, whereas 116 unigenes contained two SSRs and 29 unigenes contained three or more SSRs. Fifty-seven different SSR motif types were represented. Repeat numbers ranged from four to 42 with the average repeat length being 15.6 nucleotides.

The most predominant repeat motif found within the *M. indica* EST database were the tri-nucleotide repeats with 1,367 EST-SSRs, almost 76% of the total EST-SSRs identified (Table 4 & 5). The next most common EST-SSRs were the di-nucleotide repeats with 296 identified (16.4%), followed by tetra- (3.8%), hexa- (2.8%) and the least common penta-nucleotide repeats with just 1% found. The most frequent tri-nucleotide repeat motif was (AAG) $n$  and di-nucleotide repeat motif (AG) $n$ . This reflects the situation in grape (Scott et al. 2000), and citrus (Chen et al. 2006). Other plant types have di-nucleotide repeats as the common repeat identified, for example sugarcane (Pinto et al. 2004) and pineapple (Wöhrmann and Weising 2011). Tri-nucleotide repeats generally prevail in coding regions, which is usually attributed to selection against frame-shift mutations caused by length variation in non-trimetric repeats (Metzgar et al. 2000). Di-nucleotide repeats are typically more frequent in 5'- and / or 3'-UTR regions, but occasionally occur in coding regions as well.

### **Marker development and polymorphism of mango EST-SSRs within *Mangifera indica*.**

Only di-, tri-, tetra-, penta- and hexa-nucleotide repeats were considered as potential candidates EST-SSR marker development (Table 4) Primer pairs were designed for 36 mined EST sequences and PCR was successful for 25 with a single distinct PCR product generated across a selection of 27 *M. indica* accessions and five related *Mangifera* species. Of the 25 EST-SSR loci assessed one

marker (QGMi017) showed no polymorphism within any of the *Mangifera* species analysed. This marker was discounted in any further analyses. Only two alleles were detected for any one marker but not all loci produced allele sizes that conformed to the repeat unit length indicated. Thirteen EST-SSR markers produced allele sizes that were shorter than the repeat length of the locus (QGMi001, QGMi002, QGMi004, QGMi008, QGMi009, QGMi010, QGMi011, QGMi014, QGMi015, QGMi016, QGMi019, QGMi024 and QGMi025). A further five EST-SSR loci (QGMi006, QGMi008, QGMi019, QGMi022 and QGMi023) failed to show polymorphism at the intra-specific level within *M. indica* accessions. Discounting all six monomorphic EST-SSR loci, a total of 83 alleles were detected across the 27 *M. indica* accessions assessed (Table 3). The number of alleles detected per locus varied from two to 13 with an average of 4.37 alleles per locus. Seven EST-SSR loci had a PIC value higher than 0.5. The highest number of alleles (13) was determined for QGMi009, with a PIC value of 0.843 and the lowest number of alleles (two) was determined for QGMi007, QGMi012, QGMi014 and QGMi025. The least polymorphic was SSR loci QGMi014 with PIC value of 0.036.

Cross-species amplification of *M. indica* EST-SSR loci in five *Mangifera* species, including *Mangifera caesia*, *Mangifera foetida*, *Mangifera laurina*, *Mangifera odorata*, and *Mangifera pentandra*, was evaluated. All EST-SSR makers showed a high transferability across species. *M. caesia* showed the greatest EST-SSR loci polymorphism among analysed *Mangifera* accessions with eleven markers showing unique allele sizes in this species (Table 5), while three EST-SSR loci (QGMi010, QGMi020, and QGMi024) repeatedly failed to amplify a PCR product. *M. foetida*, *M. laurina* and *M. pentandra*, also showed unique alleles (Table 4).

Discounting the two monomorphic EST-SSR loci (QGMi007 and QGMi017) a total of 75 alleles were detected across the five *Mangifera* species assessed (Table 3). The number of alleles detected per locus varied from two to seven with an average of 3.26 alleles per locus. Eleven EST-SSR loci had a PIC value higher than 0.5. The highest number of alleles (seven) was determined for QGMi004, with a PIC value of 0.820 and the lowest number of alleles (two) was determined for QGMi006, QGMi008, QGMi014, QGMi015, QGMi018, QGMi019, QGMi020, QGMi021, and QGMi022, the least polymorphic with PIC values of 0.164. High levels of polymorphism were seen in the *Mangifera* species *M. foetida* and *M. caesia* var Binjai. The finding is in agreement with a previous diversity study of mangoes (Dillon et al. 2013). The current results are more representative as more than twice as many markers have been screened to assess polymorphisms.

The mean genetic diversity (or observed heterozygosity) for all accessions combined (*M. indica* and *Mangifera* species) was 0.360 while the mean expected heterozygosity was 0.435. The observed heterozygosity (HO) was below the expected heterozygosity (HE), indicating a tendency towards inbreeding, most likely due to population isolation. The development of 24 polymorphic EST-SSR markers increases the number of informative microsatellite markers available for *Mangifera* species. These markers will be useful for determining the genetic relationships, exploring potential pedigrees and estimating the genetic background of cultivated accessions of *Mangifera indica*.

### **Diversity analysis**

The allele data from the 25 EST-SSR markers was used to generate a bootstrapped Cavalli-Sforza distance neighbour-joining dendrogram for the 32 *Mangifera indica* and related *Mangifera* accessions (Figure 9). Cluster analysis reveals that the 32 accessions show a high level of genetic diversity, supported by the expected heterozygosity (He) value of 0.435.



Pooling the information of these 25 EST-SSR markers with data from 11 SSR markers from a previous analysis (Dillon et al. 2013) we were able to generate a bootstrapped Cavalli-Sforza distance neighbour-joining dendrogram for the 32 accessions with a total of 36 markers (Figure 9). Even with the extra 11 markers, cluster analysis continues to show a high level of diversity among the *Mangifera* accessions. The rate of polymorphism between accessions is indicative of the genetic distance among wild germplasm and commercial mango accessions in this study.

The correlation of the phenotypic data with the overall Cavalli-Sforza distance for all EST-SSR was not evident for categorical background skin, blush and pulp colours of fruit (data not shown). The development of 24 polymorphic EST-SSR markers increases the number of informative microsatellite markers available for *Mangifera* species. These markers will be useful for determining the genetic relationships, exploring potential pedigrees and estimating the genetic background of cultivated accessions of *M. indica*.

In this work we report the development of the first *M. indica* primers for amplification of polymorphic EST-SSR microsatellite loci, which are suitable to cross-amplify in five wild *Mangifera* species and useful for genetic diversity studies and other genotyping applications in *Mangifera* spp. germplasm.

**Table 2. Country of origin and phenotype colour data of 32 mango accessions used in the evaluation of EST-SSR molecular markers**

Accession	Species	Origin	Pulp colour	Background skin colour			Blush colour							
			Royal Horticulture Society colour chart	Hunter colour Scale			Colour description	Hunter colour Scale			Colour description	Hunter colour Scale		
				L.	a.	b.		L.	a.	b.		L.	a.	b.
Banana Callo	<i>M. indica</i>	Australia	Yellow/Orange (RHS15A)	62	17	52	Yellow	62	22	57	None	61	26	56
Kensington Pride	<i>M. indica</i>	Australia	Yellow/Orange (RHS23A)	64	16	50	Green/yellow	65	10	47	Orange	67	14	48
Alphonso	<i>M. indica</i>	India		69	25	56	Yellow	68	17	62	Orange	53	29	40
Creeping	<i>M. indica</i>	India	Yellow/Orange (RHS15A)	69	14	50	Green/yellow	54	-2	44	Red	53	16	39
Hybrid 17	<i>M. indica</i>	India	Yellow (RHS12A)	64	19	50	Green/yellow	66	17	54	None	70	19	56
Neelum	<i>M. indica</i>	India	Yellow/Orange (RHS15A)	69	15	47	Yellow	68	11	49	None			
Padiri	<i>M. indica</i>	India	Yellow (RHS12A)	70	8	51	Green	56	-7	31	Red	44	14	21
S. B. Chausa	<i>M. indica</i>	India	-	65	22	54	Green/yellow	61	4	42	None	40	23	7
Suvarnarekha	<i>M. indica</i>	India	Yellow/Orange (RHS15A)	69	13	55	Green/yellow	65	6	52	Burgundy	43	26	26
Apple	<i>M. indica</i>	Malaysia		67	15	53	Green/yellow	62	12	41	Orange	55	20	31
Arumanis	<i>M. indica</i>	Malesia	Yellow/Orange (RHS23A)	67	20	55	Green/yellow	65	17	56	Orange	63	21	50
Tung Chi	<i>M. indica (sens. let.)</i>	Malesia	Yellow/Orange (RHS23A)	63	19	53	Yellow	65	24	57	None	64	25	55
Carabao Lamao	<i>M. indica</i>	Philippines	Yellow/Orange (RHS15A)	62	15	52	Yellow	72	14	49	None	72	15	49
Willard	<i>M. indica</i>	Sri Lanka		66	25	54	Orange	53	6	30	Red	41	16	12
Falan	<i>M. indica</i>	Thailand	White/green	67	8	49	Yellow	73	13	57	None	74	12	55
Maha Chanook	<i>M. indica</i>	Thailand	Yellow/Orange (RHS15A)	69	18	60	Yellow	73	9	49	Pink	59	25	26
Nam Doc Mai	<i>M. indica</i>	Thailand	Yellow/Orange (RHS23A)	64	11	46	Green/yellow	63	-1	39	Orange	67	5	41
Irwin	<i>M. indica</i>	USA (Florida)	Yellow/Orange (RHS23A)	73	10	52	Green/yellow	52	12	32	Burgundy	41	25	13
Keitt	<i>M. indica</i>	USA (Florida)	Yellow (RHS9A)	64	13	48	Green/yellow	59	-2	34	Pink	53	28	24
Kent	<i>M. indica</i>	USA (Florida)	Yellow/Orange (RHS15A)	70	13	53	Green	60	4	33	Burgundy	42	35	18
Lippens	<i>M. indica</i>	USA (Florida)	Yellow/Orange (RHS15A)	67	14	51	Yellow	66	16	49	Pink	55	27	32
Palmer	<i>M. indica</i>	USA (Florida)	Yellow/Orange (RHS23A)	69	16	60	Green	55	3	51	Burgundy	36	28	11
Tommy Atkins	<i>M. indica</i>	USA (Florida)	Yellow/Orange (RHS23A)	67	14	49	Yellow	63	17	39	Burgundy	41	30	13
Van Dyke	<i>M. indica</i>	USA (Florida)	N/A	62	23	48	Pink	65	23	47	Burgundy	41	33	15
Sapa	<i>M. indica (sens. let.)</i>	Vietnam		66	13	47	Green/yellow	63	8	50	Orange	57	20	46
Xoai Cat Chu	<i>M. indica</i>	Vietnam	Yellow/Orange (RHS15A)	70	16	54	Yellow	66	-3	44	None	69	0	43
Julie	<i>M. indica</i>	West Indies		64	19	47	Green	47	10	30	Burgundy	43	23	19
Binjai	<i>M. caesia</i>	Indonesia	White	-	-	-	-	-	-	-	-	-	-	-
Bogor #2	<i>M. foetida</i>	Indonesia		-	-	-	-	-	-	-	-	-	-	-
Lomboc	<i>M. laurina</i>	Indonesia	Yellow/Orange (RHS23A)	58	17	45	Yellow	69	20	65	None	68	22	61
Unknown	<i>M. pentandra</i>	Malaysia	Yellow/Orange (RHS15A)	-	-	-	Green	-	-	-	-	-	-	-
Kweni	<i>M. odorata</i>	Malesia		66	19	60	Green	57	-3	36	None	37	-3	36

**Table 3. Characteristics of 25 EST-SSR markers screened across 27 accessions of *M. indica* and five *Mangifera* species.**

Locus	Repeat motif	Homology	Primer sequence (5' - 3')	<i>M. indica</i>					<i>Mangifera</i> species				
				Size range	No. alleles	H <sub>E</sub>	H <sub>O</sub>	PIC	Size range	No. alleles	H <sub>E</sub>	H <sub>O</sub>	PIC
QGMi001	(CCTTT)5	Short vegetative phase (flowering time)	GAAAGGCTTGCAGAGACAGG GTTTCTTCTGTTCCGGTGATGGAGGAGT	171-227	7	0.690	0.667	0.633	171-228	6	0.867	0.800	0.748
QGMi002	(CTT)4	Lacerata (CYP86A8) (plant development)	GCTCAACCTCTTTCTGCTC GTTTCTTCAATCCCCAGAAGAAAACCA	241-259	3	0.440	0.370	0.382	245-268	5	0.756	0.600	0.642
QGMi003	(CTT)6	TIR-NBS-LRR disease resistance gene	CAGGAATCTTCCCAACGAA GTTTCTTGGCAGTGTCTTCACCTTCA	157-169	4	0.516	0.556	0.445	157-169	4	0.822	0.200	0.692
QGMi004	(AAG)5	9-cis epoxycarotenoid dioxygenase (abscisic acid biosynthesis; stress response)	TTCAACAACGAGAAGACATGGA GTTTCTGGGACCTATTGATCCCACT	236-244	7	0.784	0.593	0.732	233-245	7	0.933	0.600	0.820
QGMi005	(AAC)8	WRKY40 (transcription factor; stress response)	TGGAGGAATTGAACCGATTG GTTTCTTCAGTATCGGAGGCGTCAGTC	303-318	6	0.752	0.519	0.691	303-324	4	0.733	0.600	0.610
QGMi006	(AAG)4	Squalene monooxygenase (isoprenoid biosynthesis)	GCTTGCTTCGAGTTTTTGGT GTTTCTTCGAGGAATGATCTCCGTTGT	238	1	ND	ND	ND	238-241	2	0.356	0.000	0.269
QGMi007	(ATC)5	KNAT1 (Brevipedicellus 1) (plant development)	GCCTGAAGTAGTGGCTCGAC GTTTCTTGAAATCCATGGCCTCCTGTA	307-313	2	0.073	0.074	0.069	307	1	ND	ND	ND
QGMi008	(ATC)4	WRKY7 (transcription factor; pathogen response)	TCCAGCAATTTCCACCTTTC GTTTCTTTACCATCACCAGTCAAGGA	177	1	ND	ND	ND	177-179	2	0.356	0.000	0.269
QGMi009	(AT)29	LRR transmembrane protein kinase	GGGTTAGCAAACTGGTGGA GTTTCTTCCCAAGGATATACAGTAACCAG	156-228	13	0.872	0.556	0.843	156-212	4	0.800	0.000	0.672
QGMi0010	(AGG)4	Carotenoid cleavage dioxygenase 1 (carotenoid biosynthesis)	GGTTTGAGCTTCCAAATTGC GTTTCTTCTGGGAAAGTCAACAGCAG	236-247	4	0.520	0.654	0.415	236-247	4	0.786	0.750	0.630
QGMi0011	(CCGGCT)4	Isopentenyl diphosphate isomerase 1 (isoprenoid biosynthesis)	CAACTCCGAAAGCTAGAGGAG GTTTCTTCGTGGCACTCATTACCACAC	248-290	6	0.526	0.346	0.487	248-277	3	0.511	0.600	0.410
QGMi0012	(AAG)5	UDP glucosyltransferase (flavone biosynthesis)	GGCTGAACCTAAAGGAACCA GTTTCTTATAAGCCCTCTGCCTCCAT	221-224	2	0.257	0.296	0.221	218-224	3	0.622	0.400	0.499
QGMi0013	(AAG)6	Ethylene responsive element binding factor 4 (transcription factor; stress response)	ATCAGCGTTCGGAGAGGTC GTTTCTTGCAAAAACACGAGGACCAAT	200-206	3	0.423	0.519	0.375	197-206	3	0.622	0.000	0.499
QGMi0014	(AAG)4	Arabidopsis thaliana pectin methylesterase 3 (plant development; adventitious rooting)	GCTTGCTTCGAGTTTTGGT GTTTCTTCGAGGAATGATCTCCGTTGT	214-215	2	0.037	0.037	0.036	215-216	2	0.533	0.000	0.365
QGMi0015	(AAC)7	KNAT3 (knotted1like homeobox gene 3) (plant development)	CAACCACACTTCACGGACAC GTTTCTTCATGTTTCGCTGTTGCTGT	236-247	3	0.234	0.259	0.211	236-244	2	0.200	0.200	0.164
QGMi0016	(ATCT)4	Ultrapetala 1 (plant development)	ACCAACGGCAACACCTACA	257-266	4	0.666	0.667	0.585	251-258	4	0.800	0.600	0.672

**Table 3 (cont). Characteristics of 25 EST-SSR markers screened across 27 accessions of *M. indica* and five *Mangifera* species.**

Locus	Repeat motif	Homology	Primer sequence (5' - 3')	<i>M. indica</i>					<i>Mangifera</i> species				
				Size range	No. alleles	H <sub>E</sub>	H <sub>O</sub>	PIC	Size range	No. alleles	H <sub>E</sub>	H <sub>O</sub>	PIC
QGMi0017	(CTT)6	Jasmonate insensitive 1 (RNA transcription factor)	GTTTCTTCGGCAAATCAAAGGAAAGAA GGAGAGAGTGCAGTGTCTATGG	110	1	ND	ND	ND	110	1	ND	ND	ND
QGMi0018	(AATT)5	MYB family transcription factor	GTTTCTTATTGAAGGCGTTGTTGAAGC GCTCTCTCTGTAACCTTCTTGTTT	179-195	3	0.477	0.333	0.375	183-191	2	0.533	0.400	0.365
QGMi0019	(GCT)4	Elongated hypocotyl 5 (plant development)	GTTTCTTAGTTGTGTCCGTTGTTGCTG CATGAAAAGAGATGAGGGAAA	264	1	ND	ND	ND	262-264	2	0.200	0.200	0.164
QGMi0020	(CT)7	IAA-leucine resistant 3	GTTTCTTCGGAGGCTCCAATGTAAAC GCTCTGACGCGGAGATTC	101-107	4	0.694	0.667	0.630	103-107	2	0.571	0.500	0.375
QGMi0021	(ATC)4	WRKY DNA-binding protein 15	GTTTCTTGTTGTTTTCTGGCTGCAAT GCAAGAACCAAGTGGTGTT	291	1	ND	ND	ND	291-294	2	0.356	0.000	0.269
QGMi0022	(AAC)4	MYB60	GTTTCTCCGCTGAAGAACTGAGAC CGTCTTCTCGAAGGATGGAT	157	1	ND	ND	ND	154-157	2	0.356	0.000	0.269
QGMi0023	(AAC)7	Phytochrome-associated protein 2	GTTTCTTCCTCTGTTTCTCCTCTTTCA TCAATGCAAAGAAGCTCTGAAA	133-145	5	0.734	0.926	0.676	139-145	3	0.711	0.800	0.563
QGMi0024	(GATT)4	MYB family transcription factor	GTTTCTTGCCCTCAGCTCAGTCTCCTTG CGCTTTCATCTGCTCAACTG	245-249	3	0.237	0.111	0.217	246-250	3	0.679	0.250	0.511
QGMi0025	(AGC)4	WRKY DNA-binding protein 33	GTTTCTTACACCGCCGACGCTC TAGGGAAGCACACCACGAT	300-303	2	0.465	0.333	0.352	298-303	4	0.778	0.800	0.645
			GTTTCTTGTTTCATCCTTGGCTCTCGAC										

H<sub>E</sub> = expected heterozygosity; H<sub>O</sub> = observed heterozygosity; PIC = polymorphic information content; ND = Not Determined

**Table 4. Colour categories used to evaluate the 32 mango accessions used to assess 25 EST-SSR molecular markers.**

<b>Colour category</b>	<b>Pulp colour</b>	<b>Background skin colour</b>	<b>Blush colour</b>
1	Orange (RHS25A)	green	None
2	Yellow/Orange	green/yellow	Orange
3	Yellow/Orange	yellow	Pink
4	Yellow (RHS12A)	orange	Red
5	Yellow (RHS9A)	pink	Burgundy

Bracketed date refers to the Royal Horticulture Society's colour chart code

**Table 5. Identification of EST-SSRs from Kensington Pride and Irwin.**

<b>Cultivar</b>	<b>Tissue</b>	<b>Number of Reads</b>	<b>Average length</b>	<b>Di</b>	<b>Tri</b>	<b>Tetra</b>	<b>Penta</b>	<b>Hexa</b>	<b>Total</b>
Kensington Pride	Red Leaf	6,304	473	84	347	12	3	8	454
Kensington Pride	Fruit	4,695	623	60	210	19	1	8	296
Kensington Pride	Flower	4,500	550	51	245	9	9	12	326
Kensington Pride	Root	5,302	704	39	355	22	2	20	438
Irwin	Red Leaf	4,039	564	62	210	8	4	2	286
<b>Total</b>		<b>24,840</b>		<b>296</b>	<b>1,367</b>	<b>70</b>	<b>19</b>	<b>50</b>	<b>1,802</b>

**Table 6. Allele sizes for EST-SSR loci indicating unique alleles for *Mangifera* species.**

<b>Locus</b>	<b>Unique Allele Size (bp)</b>	<b><i>Mangifera</i> species</b>
QGMi001	228	<i>Mangifera</i> sp.
QGMi002	245*, 252 <sup>#</sup> , 268 <sup>^</sup>	<i>M. caesia</i> *; <i>Mangifera</i> sp. <sup>#</sup> ; <i>M. foetida</i> <sup>^</sup>
QGMi004	233 <sup>^</sup> , 245*	<i>M. foetida</i> <sup>^</sup> ; <i>M. caesia</i> *
QGMi005	324	<i>M. caesia</i>
QGMi006	241	<i>M. caesia</i>
QGMi008	179	<i>M. caesia</i>
QGMi009	164 <sup>‡</sup> , 212 <sup>†</sup>	<i>M. foetida</i> and <i>M. odorata</i> <sup>‡</sup> ; <i>M. laurina</i> <sup>†</sup>
QGMi0011	258 <sup>#</sup> , 277 <sup>‡</sup>	<i>Mangifera</i> sp. <sup>#</sup> ; <i>M. foetida</i> and <i>M. odorata</i> <sup>‡</sup>
QGMi0012	218	<i>M. caesia</i>
QGMi0013	197	<i>M. caesia</i>
QGMi0014	216	<i>M. caesia</i> and <i>M. odorata</i>
QGMi0016	251*, 254 <sup>‡</sup>	<i>M. caesia</i> *; <i>M. foetida</i> and <i>M. odorata</i> <sup>‡</sup>
QGMi0018	183	<i>M. caesia</i> , <i>M. foetida</i> , <i>M. laurina</i> and <i>M. odorata</i>
QGMi0019	262	<i>M. caesia</i>
QGMi0020	nil	Failed to amplify in <i>M. caesia</i>
QGMi0021	294	<i>M. caesia</i>
QGMi0022	154	<i>M. caesia</i>
QGMi0024	250	<i>M. foetida</i> and <i>M. odorata</i> ; failed to amplify in <i>M. caesia</i>
QGMi0025	298 <sup>^</sup> , 301 <sup>F</sup>	<i>M. foetida</i> <sup>^</sup> ; <i>M. caesia</i> , <i>M. foetida</i> and <i>M. odorata</i> <sup>F</sup>

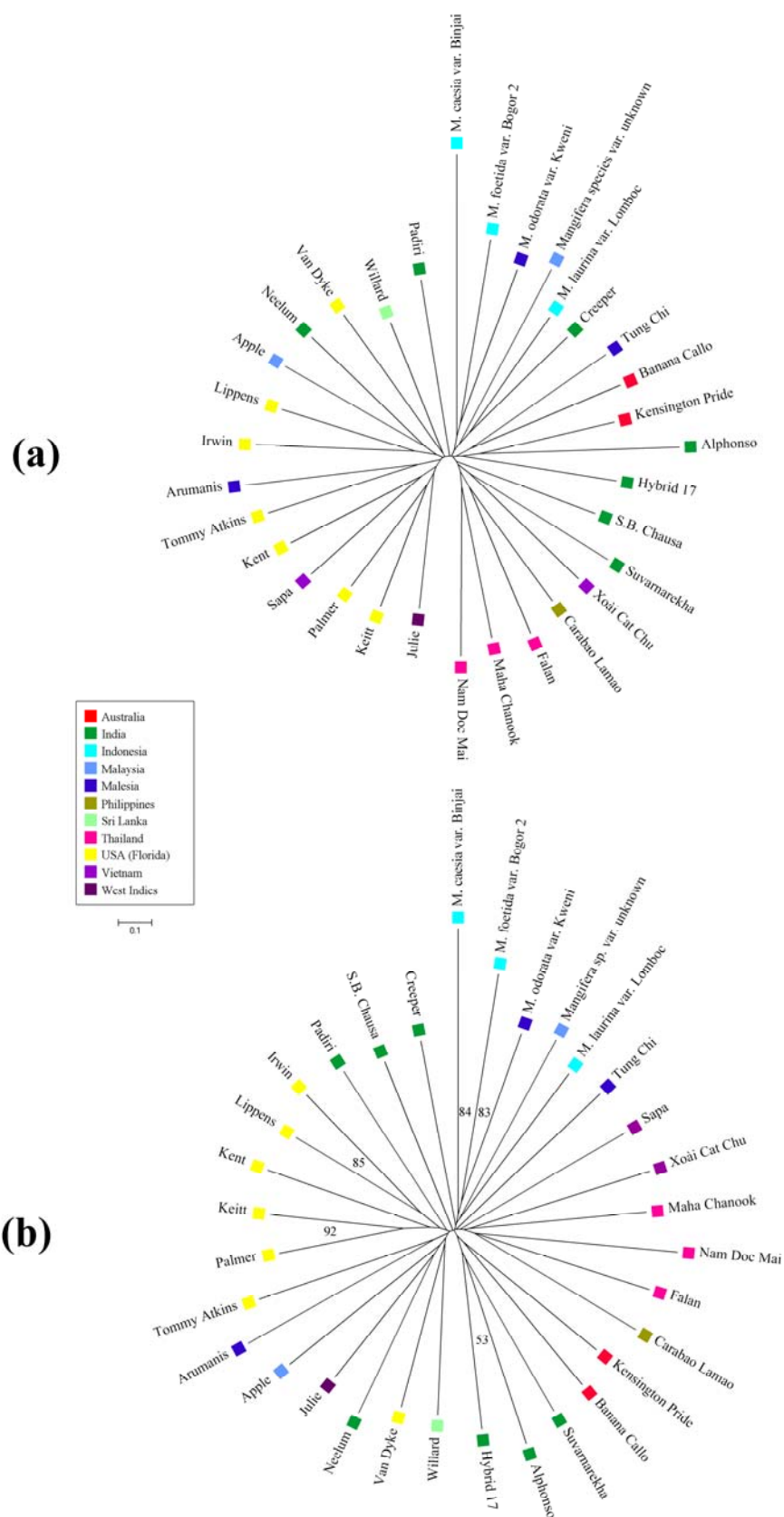


Figure 9. NJ dendrogram using Cavalli-Sforza distance based on (a) 25 EST-SSR markers and (b) 25 EST-SSR and 11 SSR markers (Dillon et al. 2010) a total of 36 markers.

### **3 Developing a recombinant linkage map**

Component Personnel: Dr Natalie Dillon

#### **Introduction**

A recombinant linkage map was planned to be developed in collaboration with the United States Department of Agriculture (USDA), Miami, USA. The map has utilised Australian mango sequence data, generated from the MFGI, that will be combined with American mango sequence data to develop SNP markers. These SNP markers will be analysed across a number of segregating hybrid families from the Australian Mango Breeding Program to produce a recombinant linkage map. The development of this recombinant linkage map will provide the foundation for the discovery of Quantitative Trait Loci (QTLs).

This work has mainly be undertaken in the USA as an in-kind contribution to the project by Dr. Kuhn who is experienced in map development in other tropical horticultural crops. In order to transfer these skills to Australian scientists, Dr. Natalie Dillon travelled to the USA in May-June 2013 to undertake training at the laboratory of Dr Kuhn.

This activity has not been completed due to several delays in Australia and the USA. In the USA Dr Ray Schnell who was the original collaborating scientist on this activity, resigned in 2011 to take up apposition with Private Industry. This delayed the start of the American activities. A more recent delay was caused by the US Government shutdown resulting in staff at the USDA being placed on furlough for a period of weeks. In Australia a -80°C freezer breakdown and subsequent loss of samples delayed activities while the insurance claim was being processed and samples recollected.

Activities have recommenced in Australia and America and the recombination linkage map will be completed as part of the follow-on HAL project 'Integrating genomics into an applied mango breeding program' (MG13002). The finalised map will be presented at the ISHS Horticultural Congress in August 2014.

#### **Materials & Methods**

##### ***Development of SNP markers***

Heterozygous sites within candidate gene sequences involved in colour and flavour development, and architecture pathways of 'Kensington Pride', 'Irwin', 'Creeper', and 'Tommy Atkins' were identified. Approximately 500 SNPs from just under 250 loci were identified sent to the USDA for Fluidigm primer and probe design. From the data 144 SNP markers were successfully developed.

##### ***Plant material***

Three Australian hybrid mango families were used in this study. 'Kensington Pride' was the male parent in these crosses including 'Creeping' x 'Kensington Pride', 'Tommy Atkins' x 'Kensington Pride' and 'Irwin' x 'Kensington Pride'.

##### ***Genomic DNA extraction***

Where possible, young fresh flushing leaf buds were collected for genomic DNA extraction. Where leaf buds were not available, the youngest leaf material available at the



time was collected. DNA extractions were performed using DNeasy® Plant extraction kits (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration and integrity was checked by analytical 1% (w/v) agarose/TBE gel electrophoresis. Extracted DNA was stored at 4°C and diluted to a final concentration of 10 ng/μl, then lyophilised prior to sending to the USA.

## **Results & Discussion**

Starting with around 250 target sequences, 103 sequences had unique gene annotations and 140 sequences had annotations that were the same as others in the set but were significantly different. It was assumed that these were different genes with different chromosomal locations. Of the 250 target sequences, around 180 of them had SNPs called at minimum read depth of 8 and a minor allele freq of 0.25. The other sequences either had insufficient depth, were poor quality, had multiple SNPs within close proximity of each other or insufficient reliable flanking sequence to be of use.

Fluidigm-designed SNP markers (144) were run across the individuals of the three hybrid families, 'Creeping' x 'Kensington Pride', 'Tommy Atkins' x 'Kensington Pride' and 'Irwin' x 'Kensington Pride'. This work was undertaken in the USA by the USDA, Miami. Of the 144 SNP markers that were run against the family 'Irwin' x 'Kensington Pride' 115 were informational. More than 20 "linkage" groups for mango were found with a number of linkage groups containing only one or two markers. To improve the genetic linkage map further SNP markers are necessary to be run across the hybrid families. It is estimated that at least a further 400 SNP markers are required.

### ***Future Plans***

This work is continuing under the new HAL project 'Integrating genomics into an applied breeding program' (MG13002). Currently the USDA has a SNP discovery project underway to identify a new selection of SNP markers. Further SNPs will be run across the Australian mango hybrid families to produce a saturated recombination linkage map for mango. This work will also utilise the vast phenotypic data collected on the Australian mango hybrid families to identify quantitative trait loci (QTLs). We plan to publish this work in a peer-reviewed journal in the near future.

## 4 Screening for anthracnose resistance and developing a leaf assay for mango

Component Personnel: Ms Kathy Grice

### Introduction and literature review

The disease anthracnose caused by the fungal organism *Colletotrichum gloeosporioides* Penz. is a major constraint to a range of horticultural industries globally (Jefferies *et al.* 1990) and mango (*M. indica*) is no exception. The organism is noted as a pre- and post-harvest problem on mango (Ploetz and Prakash 1997), especially in tropical regions where climatic conditions are conducive to disease development. Wet conditions and high relative humidity (Lim and Khoo 1985; Akem 2006) are most favourable for the spread of the organism within the tree canopy (Fitzell and Peak 1984) and for infection to occur. Pre-harvest infection occurs in the newly emerging leaves and symptoms range from small dark spots to enlarged, irregular lesions that eventually dry out and become papery. A complete blight of the young flush can also occur if the new growth and favourable weather conditions for disease development happen to coincide (Lim and Khoo 1985). Flower panicles are also very susceptible, with the organism infecting the developing flowers, which causes the flowers to die and drop off (Fitzell and Peak 1984).

Immature fruit (up to golf ball size) are also susceptible to infection and exhibit small black spots that can lead to fruit abortion. Post-harvest infection of fruit is the most severe, damaging and concerning to producers as during this phase of the disease symptoms are not expressed until fruit start to ripen (Muirhead and Grattidge 1984). These symptoms can occur anywhere along the supply chain (packing shed, transport, storage facilities or supermarket shelves) resulting in economic losses (Jeger and Plumbley 1988) to the grower.

In most mango producing countries, anthracnose is managed by an integrated programme (Lim and Khoo 1985) consisting of hygiene practices (pruning of dead material from the tree canopy), regular fungicide applications (protectant and systemic), a balanced nutrient programme and the use of post-harvest treatments such as hot water on its own or a combination of hot water and fungicide dips (Muirhead 1976; Dodd *et al.* 1991). However, even if the above treatments are carried out to a rigorous schedule, the programme is still not 100% effective at managing the disease. High quality genetic tolerance or resistance to anthracnose may be an additional strategy to managing the disease. Previous investigations by Grice *et al.* (2008), Akem *et al.* (2007) and Grice and Bally (2007) at Ayr and Mareeba used natural infection and artificial inoculation to identify a number of mango accessions with varying levels of tolerance to the disease. The wild species *M. laurina* cv. Lombok was identified as the most tolerant to *C. gloeosporioides* and has been crossed with susceptible advanced *M. indica* breeding lines.

The progeny resulting from these crosses are yet to fruit, but when they do their tolerance levels will be assessed using the artificial inoculation method to determine if the tolerance is inherited. *M. laurina* was listed by Bompard (1991) as a suitable rootstock and that no symptoms of anthracnose were observed on inflorescences when compared to other *M. indica* cultivars.

The artificial inoculation method of screening fruit for tolerance to anthracnose relies on fruit being available to test. Many seedling trees, wild *Mangifera* species and breeding

progeny have long juvenile growth stages (2-10 years) where no fruit are produced (Iyer and Degani 1997). Therefore, the time required to evaluate material can be a long drawn out process. Consequently, there is a need for the development of a rapid leaf bioassay that could be used in conjunction with the artificial fruit inoculations. This would significantly reduce the amount of time required for cultivars to be assessed by not having to wait until trees are of bearing age. However, there needs to be a strong correlation between the two methods as it is the disease tolerance levels within the fruit that are of most importance.

A search of the literature revealed that no previous research has been conducted into the development of a leaf bioassay for tolerance to *C. gloeosporioides* in mango. The only inoculation studies reported on mango were those verifying Koch's postulate to prove pathogenicity of various isolates. In these studies, a spore suspension was applied to wounded or non-wounded leaves of glasshouse grown plants (Fitzell 1979 and Gupta *et al.* 2010). In other host plant species Bigirimana and Höfte (2001) assessed various inoculation methods (seed, seedling and detached leaf) on bean (*Phaseolus vulgaris*) using the pathogen *C. lindemuthianum*. The authors found that detached leaves sprayed with a spore suspension and incubated for one week was the most suitable technique. A similar technique using detached leaves of blueberry (Ehlenfeldt *et al.* 2006) and the pathogen *C. acutatum* also gave good results. Denoyes-Rothan and Guerin (1996) screened strawberries for resistance to *C. acutatum* using six different techniques. The authors concluded that the technique of dipping whole plants in a suspension of conidia and incubating for 28 days gave the best results.

A literature review of techniques used on other pathogens was also conducted. In the screening of sour cherry germplasm against *Blumeriella jaapii* Wharton *et al.* (2003) used the inoculation methods of spraying a spore suspension on glasshouse grown plants/detached branches or a droplet of the spore suspension placed on detached leaves/leaf discs. Both methods gave similar results and the detached leaf/leaf disc method was chosen to screen a larger number of seedlings. The organism *Phytophthora palmivora* causes pod rot, a serious disease of cacao. In 2011, Santos *et al.* used detached leaves inoculated with a 200ul aliquot of spore suspension followed by an incubation period of five to seven days. This technique was used to screen >200 cacao genotypes. A number of the above techniques and variations were trialled on mango anthracnose (*C. gloeosporioides*) and are discussed in the following report.

In this project two main disease resistance components were undertaken: 1) screening for resistance to anthracnose in fruit and 2) the development of a rapid and reliable leaf bioassay to predict fruit tolerance for screening accessions at an early development stage before fruit production.

The anthracnose resistance screening trials were carried out annually to determine the resistance/tolerance of mango accessions from the Australian Mango Genebank at Southedge Research Station. The number of experiments conducted each fruiting season varied with the availability of fruit. Experiments targeted accessions that were considered to be more likely to have tolerance based on their genetic relatedness with the previously identified accession *M. laurina* cv. Lombok based on molecular phylogeny (Dillon *et al.* 2013). Three screening experiments were conducted in 2010/11, three in 2012/13 and two in 2012/2013 mango seasons.

Development of a leaf bioassay experiments were initially conducted using laboratory techniques on individual detached leaves or stems with fresh leaf flush. However, the results were not consistent enough to be reliable so one field technique was evaluated.

## **Materials & Methods**

### ***Test sample collection site***

Fruit and leaves were sourced from the Australian Mango Genebank site at the Department of Agriculture, Fisheries and Forestry (DAFF) SRS near Mareeba in north Queensland. At this site there is an outstanding collection of *M. indica* parents, hybrids and 'wild' *Mangifera* species from around the world.

### ***Pathogen isolate details***

In the preceding projects supported by Mango Fruit Genomics Initiative (MFGI) and the ACIAR Pakistan Agriculture Sector Linkages Program (ASLP), two isolates of *C. gloeosporioides* were received from the DAFF Biological Collections at Boggo Rd, Brisbane. These were pathogenicity tested on Kensington Pride mango fruit and the most aggressive isolate (BRIP 28734) was selected and used continuously throughout the life of the project. The isolate was originally obtained from anthracnose lesions evident on Kensington Pride fruit from Gin Gin in the Burnett mango-growing region.

### ***Culture storage and inoculum production***

Isolates of *C. gloeosporioides* were stored long term in a -80° freezer by placing a concentrated suspension of conidia in autoclaved 2mL tubes containing a 10% glycerol solution. To regenerate or revive the isolate for experimental use, a sterile wire loop was used to remove spores from the storage tube. This was subsequently streaked onto prepared oatmeal agar (OMA) plates. The Petri dishes were incubated at 25-26°C for 2 to 3 days (in the dark) to allow the culture to grow, then placed under near ultra-violet light (12h light/12h dark) for a further 5-10 days to induce sporulation.

Inoculum was prepared by flooding the surface of sporulating cultures (Figure 11) with sterile distilled water and dislodging the fungal spores using a sterile glass spreader. The solution was then filtered to remove unwanted mycelial fragments using 3-4 layers of muslin placed inside a conical flask. The inoculum was adjusted to a concentration of  $1-3 \times 10^6$  conidia/mL with the aid of an improved Neubauer haemocytometer (Boeco, Germany).

### ***Resistance screening (Fruit)***

#### ***Fruit collection and preparation***

The timing and number of cultivars assessed each season using the artificial inoculation method varied depending on fruits availability, maturity and quality. Eleven fruit with minimal blemishes were picked per accession at the mature hard green stage and transported back to the laboratory. The fruit were sprayed with a 70% ethanol solution, wiped with cotton wool then allowed to air dry to reduce the presence of any naturally occurring pathogens on the fruit surface. Fruit were then labelled with a code indicating the cultivar and the replication number before being placed on rubber matting, to minimise movement, inside a plastic container lined with moistened blotting paper. Approximately 1000 mL of water was placed in the bottom of the plastic container to increase the relative humidity during the incubation period. Two circles (10mm diameter) were marked on the surface of the 10 fruit to be inoculated and one non-inoculated

control fruit. In each experiment a known susceptible (Kensington Pride) and tolerant (*M. laurina* cv. Lombok) cultivar to anthracnose were included where possible as a comparative standard/reference.

### **Artificial inoculation and assessment methods**

A spore suspension of *C. gloeosporioides* was prepared from 7-14 day old cultures of isolate BRIP 28734 as previously described. Two 25 µl droplets of the spore suspension were placed in the centre of each ink marked spots on 10 fruit while the control fruit received two 25 µl drops of sterile distilled water (Figure 12). A single replication (one of each cultivar) was then packed into mango boxes before being placed in a ripening room set to 22-24 °C.

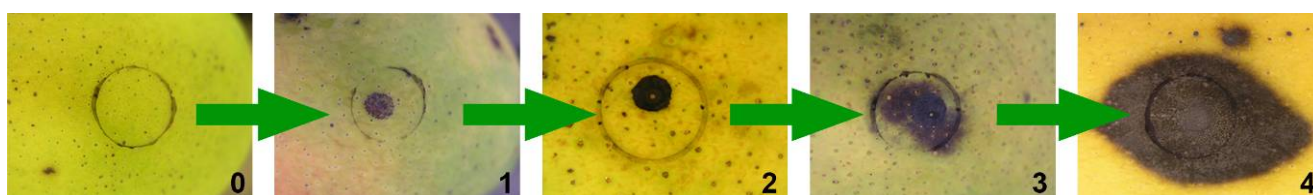
Disease development was assessed at the inoculation sites by measuring lesion diameter at five stages of fruit ripeness (1=hard green, 2=sprung, 3=sprung plus, 4=eating ripe and 5=over ripe, I Bally pers. comm.) using digital callipers. Of the five stages of ripeness, measurements at stage 4 (eating ripe) was considered the most critical as the fruit would be consumed at this stage or just prior. Fruit were also rated for disease severity using a 0–4 scale where 0=no skin discolouration; 1=skin speckling no expansion; 2=blackening of inoculation point, no expansion; 3=lesion expansion, hard; 4=lesion expansion, sunken/sporing (Figure 13). This rating was used to differentiate between cultivars that produced a hypersensitive skin reaction compared to those that developed typical anthracnose lesions.



**Figure 10.** *Colletotrichum gloeosporioides* culture growing on oatmeal agar.



**Figure 11.** Artificial inoculation of fruit using the droplet method.



**Figure 12. Progress of disease development after inoculation on a susceptible cultivar. A cultivar would be considered tolerant if development stopped at stage 2.**

### **2010/11 Season – Fruit screening - Experiment 1**

Seven accessions including Banana Callo, Kensington Pride, *M. aplanata*, *M. laurina* cv. Delphi, *M. laurina* cv. Ipoh, *M. laurina* cv. Lombok (3 selections) and *M. rubropectata* 97-865 were harvested on the 15<sup>th</sup> of November, 2010. The fruit were artificially inoculated the following day and assessed until December 4, 2010. Of the accessions tested the cv. Banana Callo was included as it was noted for its good field tolerance to the anthracnose pathogen based on screening conducted at the Ayr Research Station.

### **2010/11 Season – Fruit screening - Experiment 2**

In this experiment six accessions (13-1 Israel, Batawi, Betti Amber, Golden Delight, *M. casturi*, *M. odorata*, Manga Dodel and Sungi Siput) were evaluated in addition to the tolerant accession *M. laurina* cv. Lombok and the susceptible KP. Fruit were harvested on December 6, 2010 and the experiment was completed 21 days later. Three accessions were of particular interest (Batawi, Golden Delight and Sungi Siput) as these had performed well in the natural field tolerance experiments conducted at Ayr Research Station.

Data for the above two experiments were combined into a multi-experiment analysis using residual maximum likelihood (REML). The Wald test was used to compare the accessions, as the algorithms were not able to estimate the denominator degrees of freedom required for the F-test.

### **2010/11 Season – Fruit screening - Experiment 3**

Twelve unique accessions plus the tolerant and susceptible standards were assessed and included: Arumanis 'A', Canthamia, Chokanan 'Large', Hinchinbrook, Hybrid 17, Kent, Maha Chanook, Milika, Neelum, Padiri, Trusso and Tung Chi. The aim of this experiment was to evaluate a larger number of accessions with a reduced number of replicates (five plus an untreated control) and with minimal assessments. Based on the results, the best performing accessions would be included in a complete replicated experiment the following year. Fruit for this experiment were harvested on December 23, 2010 and the last assessment date was January 17<sup>th</sup>, 2011.

### **2011/12 Season**

Flowering and fruit set across the genebank site at SRS during this season was very poor and subsequently only eight unique accessions were evaluated.

### **2011/12 Season – Fruit Screening - Experiment 1**

Fruit of three accessions (Carabao Lamao, *M. rubropetala* 97-864 and Van Dyke) plus the standard tolerant and susceptible cultivars were picked on December 13, 2011 and assessed for the following 26 days.

### **2011/12 Season – Fruit Screening - Experiment 2**

The accessions Asam Ramuk, Cheneke, Creeper, Hong Sa, and *M. pandandra* plus the susceptible standard KP were included in this experiment. Fruit were harvested on January 10, 2012 and assessed until February 1, 2012.

### **2012/13 Season**

#### **2012/13 Season – Fruit screening - Experiment 1**

This experiment was conducted over the period December 12, 2012 to January 5, 2013 using the following accessions: 20/26, Ampalam, Carabao 1, Jewel, *M. aplanata* 'Pilipisan', *M. laurina* cv. Lombok (3 selections of the tolerant standard), Raet and Tung Chi. The susceptible standard KP was not included in this experiment as the fruit were immature.

#### **2012/13 Season – Fruit screening - Experiment 2**

The following nine accessions: 20/26, Arumanis Red, Asam Ramuk, BOT, Carrie, Creeper, *M. quadrafida*, Mangee Dodel and Tung Chi were screened in this experiment along with the susceptible standard KP. *M. laurina* cv. Lombok, the tolerant standard was not included as all the fruit available were used in the previous experiment. Because of this, two cultivars 20/26 and Tung Chi tested in the previous experiment were reassessed to give some continuity. Fruit were collected on 2 January, 2013 and the experiment ceased on 27 January, 2013.

#### **2012/13 Season – Fruit screening - Experiment 3**

In the last experiment for the season, 13 accessions (20/26, B5, Duncan, Haem Wangi, Hybrid 17, Kensington Pride, Keow, Maha Chanook, Padiri, Rajah, Trusso, Van Dyke and Xoai Bu) were assessed. As with the previous two experiments, only one standard (KP) was available for inclusion, therefore the accession 20/26 was again reassessed. Fruit were harvested on January 29, 2013 and the experiment ceased on February 19, 2013.

### **Statistical analysis**

The mean lesion diameter of each fruit at the five ripeness stages was analysed using residual maximum likelihood (REML). As different numbers of fruit were recorded at each ripeness stage, REML allows for unbalanced data. A log<sub>10</sub> transformation of the data was required prior to some analyses and due to zero mean diameters, a small constant was added before making the transformation. Where a transformation was required, the means are presented on both the transformed and back-transformed (italics) scales. Where the F-probability (p-value) was significant (<0.05), pairwise comparison were made using the average 95% least significant difference (LSD), except in 2011/12 season when it was possible to use the actual 95% LSDs due to only a small number of accessions being tested. In general, the average is used for statistical simplicity rather

than making a large number of comparisons with the individual LSDs. Depending on the number of experiments conducted during a season and whether there were commonalities between accessions some combined analyses were performed. For some data sets, the Wald test was used to compare the cultivars rather than the F-test. This is due to the algorithms inability to estimate the denominator degrees of freedom for the F-test. Analyses were conducted using GenStat 11 (GenStat 2008) and GenStat for Windows 13<sup>th</sup> Edition (VSN International 2010).

### **Leaf bioassay for anthracnose tolerance**

#### **Leaf disc (laboratory method)**

A number of laboratory experiments were conducted using leaf discs of various sizes cut from a range of leaf ages of pink and green flush of the accessions Kensington Pride and *M. laurina* cv. Lombok. Three inoculation techniques were assessed. (1) A 25 µl droplet of spore suspension (concentrations  $10^5$ ,  $10^6$  and  $10^7$ ) were deposited on the upper leaf surface. (2) Plugs (5 mm diam.) of actively growing *C. gloeosporioides* culture were placed on the upper leaf surface with the mycelium in contact with the leaf. (3) Whole leaf discs were immersed in the spore suspension for 30 seconds then allowed to dry.

Some of the treatments were also applied to leaves with and without wounding (discs pierced with a sterile needle). All of the above techniques were conducted in the laboratory in glass Petri dishes. A layer of glass beads (5mm) was placed in the bottom of each Petri dish to support the leaf discs above a benzimidazole solution (50mg/L), which was used to inhibit or reduce the rate of senescence (Mishra and Misra 1973).

#### **Detached leaf flush (laboratory method)**

Two individual experiments were conducted in the laboratory using detached mango flush from four accessions: *M. laurina* cv. Lombok, Kensington Pride, *M. rubropetala* 97-864 and Keitt. Newly produced flush, 4 stems per cultivar were collected from trees at SRS and returned to the laboratory. A stock suspension ( $10^6$ ) was prepared from 7-10 day axenic cultures of *C. gloeosporioides* using a haemocytometer and subsequently diluted 1:10 and 1:100 to produce suspensions at  $10^5$  and  $10^4$ .

#### **Detached leaf flush (laboratory method) - Experiment 1**

In this experiment, the accessions *M. laurina* cv. Lombok, Kensington Pride and *M. rubropetala* 97-864 were collected from SRS on July 24, 2012. Stems were re-cut before being placed in beakers containing florist oasis<sup>®</sup> and sterile distilled water. A stem with leaf flush was sprayed until runoff with one of the spore suspensions using an atomiser, then bagged for 48 hours (Figure 14). One flush was sprayed with sterile distilled water and used as the control. After 48 hours of incubation the bags were removed and the flush monitored for disease development over the coming days.

#### **Detached leaf flush (laboratory method) - Experiment 2**

On August 1, 2012, the accessions *M. laurina* cv. Lombok, Kensington Pride and Keitt used for this experiment were collected. Stems with fresh leaf flush were submerged in cold water for one hour to help prevent water loss and wilting (P. Trevorrow pers. comm.). Stems were then removed and recut before being inserted into the oasis<sup>®</sup>. On this



occasion the beakers contained a solution of benzimidazole (50mg/L) to slow the rate of senescence. The beakers were also placed on a tray filled with water to increase the humidity in an air-conditioned environment. The treatments applied were the same as those in experiment 1. After 48 hours, the bags were removed and the flush was sprayed intermittently throughout the day with sterile distilled water to keep the leaves fresh and moist.

### ***Leaf flush (field method)***

Two experiments with similar treatments to those used in the detached leaf flush (laboratory method) were conducted in the field at SRS.

### ***Leaf flush (field method) - Experiment 1***

This experiment was initiated on 1 August, 2012 and was conducted in conjunction with the second detached leaf flush (laboratory method) experiment using the same accessions. One leaf flush was inoculated per inoculum concentration in addition to an untreated control. Plastic bags were misted with water then placed over the inoculated leaf flush and sealed for 48 hours to maintain high humidity (Figure 15). The leaf flush was cut from the tree using secateurs nine days after inoculation and assessed for disease symptoms (% area infected per leaf).

### ***Leaf flush (field method) - Experiment 2***

On August 27, 2012, a second experiment was conducted using the same technique as mentioned above but with an increased number of accessions including: KP (2 trees), *M. indica* cv. Lombok, Hybrid 17, Gudang, Lippens and Neelum.

### ***Spray inoculated leaf flush (in field) method***

Two experiments with similar treatments to those used in the detached leaf flush method were conducted in the field at SRS. The first experiment was conducted in conjunction with laboratory experiment 2 using the same cultivars, one leaf flush per inoculum concentration and an untreated control. Plastic bags were misted with water then placed over the inoculated leaf flush and sealed for 48 hours to maintain high humidity (Figure 15). The leaf flush was cut from the tree using secateurs nine days after inoculation and assessed for disease symptoms (% area infected per leaf).

A second experiment was conducted using the same technique as mentioned above but with an increased number of cultivars including: Kensington Pride (2 trees), *M. indica* cv. Laurina., Hybrid 17, Gudang, Lippens and Neelum.



Figure 13. Detached flush inoculated with *C. gloeosporioides* in the laboratory.



Figure 14. Flush on the cultivar Keitt inoculated with *C. gloeosporioides*.

## Results

### 2010/11 Season - Fruit screening - Experiment 1 and 2

The results from the combined analysis (Table 7) indicated that the *M. laurina* cv. Lombok selections (1-3, Figure 17), along with 13-1 Israel and Betti Amber had consistently smaller mean lesion size at ripeness stage 4. The next best accession was *M. rubropetala* 97-864, yet there were no significant differences between eleven of the remaining accessions tested. The accessions that had the largest mean lesion diameter at ripeness stage 4 included: Banana Callo (Figure 17), Batawi, Golden Delight and *M. casturi*. The disease severity rating data has not been tabled in this report, however, the cultivars with the lowest severity rating were the same as those listed above and also included *M. casturi* and *M. rubropetala* 97-864 all with mean rating of less than 1.0.



Figure 16. Disease development 14 days post inoculation.



Figure 16. Disease development 14 days post inoculation.

### **2010/11 Season - Fruit screening - Experiment 3**

As the fruit were not assessed at each ripeness stage as in previous experiments, the only data that could be analysed and presented was ripeness stage 5 (Table 8). There were no significant differences between the accessions Arumanis 'A', Kent, Milika, Neelum, Padiri or Trusso that recorded the lowest mean lesion diameters of <9mm. On this occasion the tolerant accession *M. laurina* cv. Lombok did not typically record the lowest mean lesion diameter but it was not significantly different to four of the accessions already listed. The accession that recorded the largest mean diameter (>27mm) was Chokanan 'Large' but it was not significantly different to six other accessions.

### **2011/12 Season - Fruit screening - Experiment 1**

Of the six *M. laurina* cv. Lombok fruit available for testing, it was noted that three were already at ripeness stage 2 prior to being artificially inoculated. The results from the analysis of mean lesion diameters at each ripeness stage are presented below in Table 9. At ripeness stage 4 there were no significant differences between the cultivars Carabao Lamao, *M. laurina* cv. Lombok or *M. rubropetala* 97-864. The cultivar with the largest average lesion diameter (>13mm) at ripeness stage 4 was Van Dyke. The overall results for the disease severity rating correlated with the mean lesion diameters at ripeness stage 4 as stated above and are therefore not tabled.

Table 7. Mean lesion diameters of mango fruit artificially inoculated with *C. gloeosporioides* and assessed at four stages of ripeness.

Cultivar	Ripeness 2		Ripeness 3		Ripeness 4		Ripeness 5	
	Transformed d	Bt(mm)	Transformed	Bt(mm)	Transformed	Bt(mm)	Transformed	Bt(mm)
13-1 Israel	-2.001 a	0.000	-1.551 ab	0.018	-0.654 a	0.212	0.406 ab	2.536
Banana Callo	-1.206 b	0.052	0.282 ef	1.903	0.912 c	8.150	1.320 c	20.887
Batawi	-1.760 a	0.007	0.460 efg	2.874	0.966 c	9.241	0.993 bc	9.830
Betti Amber	-2.015 a	0.000	-1.266 b	0.144	-0.714 a	0.183	0.393 ab	2.462
Golden Delight	-0.610 cd	0.236	0.716 fg	5.191	1.108 c	12.825	1.154 c	14.255
Kensington Pride	-0.066 e	0.849	0.176 de	1.489	0.420 bc	2.618	0.874 bc	7.476
<i>M. aplanata</i>	0.701 f	5.009	0.809 g	6.431	0.727 bc	5.320	0.930 bc	8.494
<i>M. casturi</i>	0.454 f	2.838	0.605 efg	4.017	0.990 c	9.767	0.937 bc	8.645
<i>M. laurina</i> cv. Lombok (1)	-2.001 a	0.000	-1.480 ab	0.023	-0.806 a	0.146	-0.197 a	0.625
<i>M. laurina</i> cv. Lombok (2)	-2.007 a	0.000	-1.635 ab	0.013	-1.169 a	0.058	-0.064 a	0.853
<i>M. laurina</i> cv. Lombok (3)	-2.001 a	0.000	-1.906 a	0.002	-1.319 a	0.038	-0.200 a	0.621
<i>M. laurina</i> - Delphi	-2.001 a	0.000	-0.239 cd	0.567	0.412 bc	2.575	1.138 c	13.718
<i>M. laurina</i> - Ipoh	0.278 ef	1.889	0.453 efg	2.826	0.399 bc	2.494	0.916 bc	8.239
<i>M. odorata</i>	-1.777 a	0.007	-0.353 c	0.434	0.672 bc	4.686	0.953 bc	8.968
<i>M. rubropetala</i> 97-864	-0.196 de	0.626	0.179 de	1.499	0.154 b	1.415	0.803 bc	6.345
Manga Dodel	0.256 ef	1.792	0.513 efg	3.245	0.801 bc	6.315	0.888 bc	7.717
Sungi Siput	-1.092 bc	0.071	0.520 efg	3.303	0.807 bc	6.407	0.795 bc	6.232
p-value	<0.001		<0.001		<0.001		<0.001	
Average SED	0.2626		0.2253		0.3770		0.3680	
Average LSD	0.5185		0.4449		0.7443		0.7272	
df	16		16		16		16	

Means within a column followed by the same letter are not significantly different.

Bt – Back transformed means expressed in millimetres.

**Table 8. Mean lesion diameters of mango fruit artificially inoculated with *C. gloeosporioides*.**

Cultivar	Ripeness 5*	
	Tranformed	Back transformed (mm)
Arumanis 'A'	0.848 a	7.043
Canthamia	1.165 bcdefg	14.626
Chokanan (Large)	1.437 g	27.369
Hinchinbrook	1.218 defg	16.492
Hybrid 17	1.177 cdefg	15.032
Kensington Pride	1.399 fg	25.062
Kent	0.952 abcd	8.935
<i>M. laurina</i> - D	1.142 bcdef	13.865
MacLeod		
Maha Chanook	1.262 efg	18.288
Milika	1.042 abcde	11.001
Neelum	0.889 ab	7.742
Padri	0.904 abc	8.007
Trusso	0.825 a	6.681
Tung Chi	1.384 fg	24.192
p-value	<0.001	
Average SED	0.1386	
Average LSD	0.2800	
df	40.9	

\*Only ripeness stage 5 had enough data available for a valid analysis.

Means followed by the same letter are not significantly different.

**Table 9. Mean lesion diameters of mango fruit artificially inoculated with *C. gloeosporioides* and assessed at four stages of ripeness**

Cultivar	Ripeness 2	Ripeness 3		Ripeness 4		Ripeness 5
	Mean (mm)	Transform ed	Bt(mm)	Transform ed	Bt(mm)	Mean (mm)
Carabao Lamac	0.884 a	0.377 a	2.383	0.620 a	4.173	8.112 ab
Kensington Pride - Fordice	3.400 b	0.594 b	3.926	0.759 b	5.738	10.179 b
<i>M. laurina</i> cv. Lombok	1.098 a	0.485 ab	3.058	0.630 ab	4.267	9.575 b
<i>M. rubropetala</i> 97-864	3.220 b	0.610 b	4.069	0.615 a	4.124	5.413 a
Van Dyke	3.384 b	0.879 c	7.564	1.143 c	13.892	18.400 c
p-value	<0.001	<0.001		<0.001		<0.001
Average SED	0.6838	0.0778		0.0626		1.9034
Average LSD	1.3961	0.1586		0.1265		3.8501
df	30.2	31.9		39.0		39.0

Means within a column followed by the same letter are not significantly different.

Bt – Back transformed means expressed in millimetres.

### **2011/12 Season - Fruit screening - Experiment 2**

It was noted during the course of the experiment that of the eleven Cheneke fruit harvested, five succumbed to fruit fly damage and had to be discarded. Observations were also made that disease symptoms did not develop at the normal rate in all accessions except one (Creeper). It was later discovered that a pre-harvest application of the fungicide Amistar® was applied without our knowledge prior to fruit harvest. Creeper, the accession that did develop anthracnose symptoms, was a drooping dwarf type and it is suspected that the foliage would have protected the fruit from the fungicide application. Due to this information and the lack of disease symptoms expressed on the other accessions, the results of this experiment were discarded and are not tabled in this report. All the accessions used in this experiment need to be reassessed when fruit are available.

### **Season 2012/13**

Three separate experiments using the artificial inoculation technique were conducted in the 2012 to 2013 season on a total of 26 cultivars. The mean diameter measurements for all experiments were analysed separately using REML as well as in a combined analysis. The results for all three experiments conducted in this season are shown in Table 10 using the combined analysis.

### **2012/13 Season - Fruit Screening - Experiment 1, 2 and 3**

In experiment 1 and 2 it was noted that the accession 20/26 was quite slow to ripen compared to most of the other accessions indicating that it may have been immature. In experiment 1, both Jewel and Raet also appeared to be immature as ripening was delayed, however, disease development did not seem to be hampered (Table 10). In the combined analysis the accessions considered to be tolerant based on the mean lesion diameter being <5mm at ripeness stage 4 were all the *Mangifera* species plus Ampalam, BOT, Carabao 1, Carrie, Mangee Dodel and Raet. At the other extreme, the accessions considered to be susceptible included Creeper, Hybrid 17, Jewel and Van Dyke recording mean lesion diameters of >10mm.

### **Leaf Bioassay for Anthracnose Tolerance**

#### **Leaf disc (laboratory method)**

All conidial suspensions applied as a droplet to leaf discs of the cultivar KP irrespective of concentration caused infection across a range of leaf ages with no obvious differences. There were also no differences whether the flush was pink or green in colour (Figure 17). The results of the following three techniques (droplet- $10^6$ , mycelial plug and disc dip) applied to Kensington Pride and *M. laurina* cv. Lombok was variable. All leaf ages across both accessions were susceptible when the disc dip method was used. Having a point of injury allowed the disease to progress rapidly. The result of the mycelial plug and droplet method varied and there didn't appear to any pattern to whether infection occurred or not.

**Table 10. Mean lesion diameters of mango fruit artificially inoculated with *C. gloeosporioides* and assessed at four stages of ripeness.**

Accession	Ripeness 1	Ripeness 2	Ripeness 3		Ripeness 4		Ripeness 5	
	Mean (mm)	Mean (mm)	Transformed	Bt (mm)	Transformed	Bt (mm)	Transformed	Bt (mm)
20/26	3.479 k	4.790 hijkl	0.828 kmno	5.731	0.961 hijkl	8.136	1.163 f hijk	13.542
Ampalam	0.000 a	0.000 a	0.400 a	1.512	0.723 ab	4.288	1.087 efgh	11.214
Arumanis red	3.186 jk	5.037 hijkl	0.849 kmnop	6.060	1.008 ijklm	9.188	1.133 efghij	12.585
Asam Ramuk	0.657 bc	0.291 a	0.662 b	3.593	0.865 efg	6.332	1.114 efghi	11.988
B5	3.076 hijk	4.823 ghijkl	0.835 kmno	5.836	0.994 klm	8.869	1.191 ghijkl	14.530
BOT	2.712 ghijk	3.456 cdefg	0.729 bcdefghi	4.352	0.739 abc	4.477	0.827 ac	5.713
Carabao 1	1.422 cdefgh	3.910 defghi	0.744 bcdefghijk	4.542	0.715 ab	4.191	0.905 abc	7.033
Carrie	0.872 cd	3.114 bcdef	0.676 bc	3.747	0.751 abcd	4.640	0.820 ab	5.605
Creeper	1.627 cdefghi	3.124 bcdef	0.940 pq	7.704	1.210 n	15.225	1.281 fghijklm	18.089
Duncan	2.816 hijk	3.591 defgh	0.778 d fghijklmno	5.003	0.874 defghi	6.475	0.970 abcde	8.340
Haem Wangi	2.408 fghijk	3.875 efgh	0.796 d fghijklmno	5.252	1.012 lm	9.290	1.173 ghijkl	13.905
Hybrid 17	5.118 l	5.767 l	0.970 q	8.337	1.219 n	15.558	1.462 m	28.005
Jewel	2.505 fgijk	5.794 i kl	1.133 r	12.573	1.259 n	17.168		
Kensington Pride	5.099 l	4.689 ghijkl	0.812 g ijklmno	5.482	0.989 klm	8.745	1.23 i jkl	15.998
Keow	2.774 hijk	4.791 ghijkl	0.823 g jklmno	5.656	0.986 g jklm	8.679	1.204 ghijkl	15.002
<i>M. aplanata</i> -Pilipisan		4.955 ghijkl	0.786 bcdefghijklmno	5.108	0.742 abc	4.516	0.942 bcd	7.746
<i>M. laurina</i> cv. Lombok (1)	0.000 a	1.623 ab	0.709 bcdefg	4.111	0.692 ab	3.922	0.796 a	5.256
<i>M. laurina</i> cv. Lombok (2)	0.000 ab	1.617 ab	0.679 bcd	3.770	0.681 ab	3.800	0.894 abc	6.835
<i>M. laurina</i> cv. Lombok (3)	0.000 a	1.684 abc	0.683 bcdef	3.814	0.676 a	3.738	0.845 ab	5.995
<i>M. quadrafida</i>	1.661 cdefhi	4.119 efghijk	0.732 bcdefghij	4.394	0.760 abcde	4.750	0.886 abc	6.695
Maha Chanook	0.979 cde	2.322 bcd	0.810 g ijklmno	5.460	1.006 lm	9.147	1.314 j lmn	19.603
Mangee Dodel	1.217 cdef	3.357 bcdefg	0.713 bcdefgh	4.166	0.737 abc	4.463	0.858 abc	6.209
Padiri	2.426fghijk	2.877bcde	0.680bcde	3.783	0.895 fghijk	6.846	1.027 cdef	9.643
Raet	2.503fgijk	4.506 efghijkl	0.767 bcdefghijklm	4.842	0.760 bcde	4.759	0.964 bcde	8.206
Rajah	3.036 hijk	4.444 fghijkl	0.768 cd fghijklmn	4.861	0.869 defgh	6.394	0.944 abcde	7.785
Trusso	5.414 l	5.098 hijkl	0.839 k mno	5.906	0.971 ghijklm	8.349	1.148 fghijk	13.046
Tung Chi	1.243 cdefg	3.985 efghij	0.744 bcdefghijkl	4.543	0.809 cdef	5.442	1.067 efg	10.667
Van Dyke	2.261 d fghij	4.755 ghijkl	0.858 k m op	6.218	1.073 m	10.825	1.294 ijklmn	18.700
Xoai Bu	2.417 defghijk	3.830 defgh	0.773 d fghijklmn	4.929	0.887 fghij	6.710	1.092 defgh	11.362
p-value	<0.001	0.022	<0.001		<0.001		<0.001	
Average SED	0.7264	0.5892	0.0542		0.0566		0.0834	
Average LSD	1.431	1.6360	0.1068		0.1116		0.1644	

Means within a column followed by the same letter are not significantly different. Bt – back transformed means expressed in millimetres



### ***Detached leaf flush (laboratory method)***

Both of the spray inoculated detached leaf flush experiments conducted suffered the same plight. The flush used in the first experiment started to wither and dry out before any disease symptoms were observed (three days post inoculation). The leaf flush used in the second experiment lasted one day longer, even though the water was replaced by a solution of benzimidazole. Based on the results of both experiments, this technique was not appropriate as the young tissue was too sensitive when removed from the tree and could not be kept alive for any length of time.

### ***Leaf flush (field method)***

Lesions developed on the susceptible cultivar Kensington Pride at the inoculation concentrations of  $10^4$  and  $10^5$  (Figure 17). Unfortunately, the leaf flush inoculated with the high concentration ( $10^6$ ) was broken off the tree when the plastic bag was removed. No lesions developed on *M. laurina* cv. Lombok or Keitt, the former is known to have fruit tolerance to *C. gloeosporioides* while Keitt is supposed to be moderately tolerant. No results were obtained from experiment two due to unforeseen field applications of fungicide applied the day the inoculations took place.

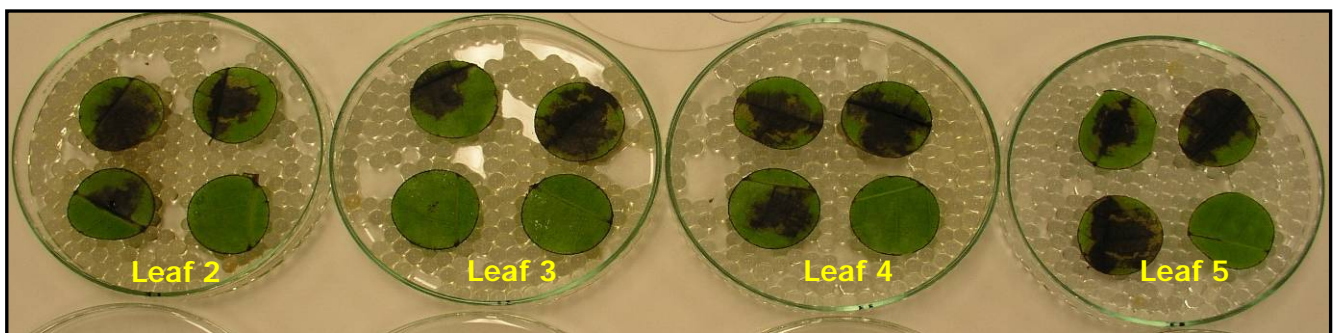


Figure 17. Leaf disc inoculations on green flush using the droplet method on Kensington Pride. From the bottom left of each dish treatments are:  $10^5$ ,  $10^6$ ,  $10^7$  and sterile distilled water



Figure 18. Flush spray inoculated in the field at the concentration of  $10^5$ . *M. laurina* cv. Lombok (left) and KP (right)



## Discussion

More than 105 mango accessions (parents, hybrids and *Mangifera* species) have been screened for their susceptibility to the fungal organism *C. gloeosporioides* in this and previous projects. This has been achieved through natural infection and artificial inoculation techniques. A number of cultivars (Banana Callo, Batawi, Golden Delight and Sungi Siput) which were previously assessed as being tolerant based on natural infection levels were subjected to the artificial inoculation test and succumbed to infection. Based on these assessments, no mango accession has expressed total resistance but some accessions show a degree of tolerance to the disease. To date, the wild species *M. laurina* cv. Lombok has shown the most promise with the lowest rate of disease development over a number of seasons. However, the level of tolerance varies from year to year and may be due to climatic conditions experienced throughout the growing season.

Crosses have been made between the tolerant species *M. laurina* cv. Lombok and some elite *M. indica* lines from the Australian Mango Breeding Program (AMBP). The status of this progeny will be evaluated once the trees reach fruiting stage to determine if the tolerance has been inherited. There are other cultivars including Betti Amber and 13-1 that have shown promise based on the initial testing, however, fruit for further evaluations has not been available in recent years.

In the 2010/11 season the results of (Fruit Screening - Experiment 3), indicated that a reduced number of replicates and limited assessments were not adequate to differentiate between accessions. A statistical analysis could only be performed on ripeness stage 5 and by this stage disease development was quite advanced. However, there were still some positives that could be taken from this information, mainly those accessions that can be discarded from future assessments.

There were a number of downsides to the artificial inoculation and other methods of screening mango fruit for their tolerance or susceptibility to the fungal organism *C. gloeosporioides*. The test relies on the trees to bear fruit, unfortunately there was no way of manipulating trees to ensure that they produce the number and quality of fruit required, therefore screening with this method can be a lengthy process. The genetic diversity in the genebank at Southedge Research Station is extensive and the testing was very dependant upon fruit being at the same stage of maturity. There have been issues with on-farm practices (fungicide application in particular) being applied to all sites at the SRS which has compromised the trial work being conducted.

The development of a leaf assay to determine the tolerance or susceptibility of mangoes to *C. gloeosporioides* has been difficult and challenging. The laboratory method using leaf discs based on the techniques used was unsuitable due to the inconsistent results. The detached leaf flush method failed, as it was not possible to keep the material alive for more than 4-5 days in the laboratory and the timeframe was not long enough to observe any symptoms of disease development. There are other methods using detached whole leaves that may prove to be a better option and could be pursued further in another project.

The inoculation of leaf flush in the field is definitely an option to pursue as early results in this project using Kensington Pride and *M. laurina* cv. Lombok showed promise with the susceptible cultivar developing symptoms and the tolerant accession being free of disease symptoms. Further testing of this method needs to be conducted before any comparisons

on leaf and fruit tolerances can be established and before a leaf bioassay can reliably replace the artificial inoculation testing of fruit. It is highly possible that the resistance mechanism between the fruit and the vegetative material are completely different pathways and will result in no correlation.

## **Future activities**

The assessment of the anthracnose disease tolerance/susceptibility status of the hybrid progeny will be evaluated as the trees mature and crop as part of the HAL project 'Integrating genomics into an applied mango breeding program' (MG13002).

## **5 Develop test hybrid populations to use in genetic and genomic studies of disease resistance in mango**

Component Personnel: Dr. Ian Bally, Ms Cheryl Maddox

### **Introduction**

Previous investigations by Grice et al. (2008), Akem et al. (2007) and Grice and Bally (2007) at Ayr and Mareeba used natural infection and artificial inoculation to identify a number of mango accessions with varying levels of tolerance to anthracnose. The wild species *M. laurina* cv. Lombok was identified as the most tolerant to the pathogen *C. gloeosporioides*. To improve our understanding of this tolerance, discover the genes involved and its transferability to more commercial cultivars, a test hybrid population is required where the disease tolerant accession is crossed with susceptible more commercial cultivars.

This project aimed to build up a hybrid population that is segregating for anthracnose tolerance that can be used to study natural resistance to anthracnose in mango. As the mango breeding process is slow, it is expected that several years of crossing will be required before a large enough population is established for genetic and genomic studies. When these progeny mature and fruit they will be assessed for anthracnose tolerance.

### **Materials & Methods**

The anthracnose tolerant accession *M. laurina* cv. Lombok is a polyembryonic seeded type that can only be used as a male or paternal parent in a hybridisation program.

Pollen from this accession growing on DAFF's Southedge Research Station was used in controlled hand pollination of three advanced breeding lines (Figure 20) from the Australian Mango Breeding program. The pollination process followed was described by Bally et al. (2009) and was conducted over three seasons (2010 to 2012).

## Results & Discussion

Over the three seasons 1016 flower panicles were hand pollinated using pollen from the anthracnose tolerant accession *M. laurina* cv. Lombok which resulted in 58 hybrid seedlings (Table 11). These progeny have been planted on DAFF's Walkamin Research Station for growth until maturity when fruit will be available for anthracnose tolerance testing. To date no progeny have fruited, but the oldest ones may begin in the 2013-2014 season. Development of this segregating disease population will continue beyond this project. The assessment of the tolerance/susceptibility status of the hybrid progeny will be evaluated as the trees mature and crop as part of the HAL project 'Integrating genomics into an applied mango breeding program' (MG13002).

**Table 11. Parents, pollinations and progeny produced from crossing the anthracnose tolerant *M. laurina* cv. Lombok and three breeding lines from 2009 to 2012.**

Female Parent	Male Parent	2009		2010		2011		2012		total
		Number of Pollinations	Hybrids	Number of Pollinations	Hybrids	Number of Pollinations	Hybrids	Number of Pollinations	Hybrids	
10035	<i>M. laurina</i>	-		45	3	-	-	-	-	3
1243	<i>M. laurina</i>	5	0	360	36	-	-	218	7	43
4046	<i>M. laurina</i>	302	57	299	6	-	-	94	6	69



NMBP 1243



NMBP 4046



NMBP 10035



*M. laurina* cv. Lombok

Figure 19. Mango cultivars (top 3 photos) hybridised to *M. laurina* cv. Lombok (bottom 2 photos) with tolerance to postharvest anthracnose disease.

## Technology Transfer

The main audience for the technologies developed in this project are research organisations and scientists involved with breeding and genetic improvement of mangoes and other tropical fruit. The main recipient of the technology is the Australian Mango Breeding Program where the research of this project was undertaken. The success of adoption of these technologies into the mango breeding program has been dependant on their stage of development and practical advantages over currently used technologies. Several of the genomic and pathology technologies developed in this project are not yet fully developed and not at a stage where they can be routinely used in the mango breeding program.

One of the successful discoveries that have been adopted into the mango breeding program is the incorporation of anthracnose postharvest disease resistance into the breeding program. No disease tolerant progeny have cropped and been tested to date. The importance and consequences of having natural disease resistance to postharvest anthracnose has been recognised and adopted as a major goal of the Australian Mango Breeding program.

Dr. Natalie Dillon, a key researcher in this project, was the recipient of knowledge and training from the United States of America when she spent three weeks training in the Laboratory of Dr David Kuhn of the USDA's Agriculture Research Services (ARS) Subtropical Horticulture Research Station (SHRS) in Miami. Dr Dillon trained on the latest SNP technologies and in the development of molecular recombination maps. She learnt basics of running Fluidigm SNP markers, calling SNP marker results, the use of the software JoinMap to develop a recombination linkage map for mango. Dr Dillon also developed the collaboration between the USDA and DAFF, furthering chances of future collaborative research in the field of mango genomics.

Throughout the project the Australian Mango Industry and other interested scientists have been kept abreast of developments through publications, seminars and presentations given by project staff at national and international conferences as listed below:

### ***Seminar and conference and field day presentations***

Bally ISE, Akem CN, Dillon NL, Grice K, Lakhesar D, Stockdale K (2013) Screening and breeding for genetic resistance to anthracnose in mango. *Acta Horticulturae* **992**, 239-244.

Bally ISE (2012) New Mango Cultivars, Northern Territory Mango Research and Extension Forum 15 March 2012.

Bally ISE (2012) Mango Breeding Field day, Southedge Research Station, Mareeba, QLD, 19/12/2012

Bally ISE (2012)– Mango Breeding Field Day, Frank Wise Institute, Kununurra, WA, 18/10/2012

Bally ISE, Johnson PJ, Henroid R (2011) Mango Breeding. In '8th Australian Mango Conference.' (AMIA: Darwin, Australia, 18-21 April 2011)

Bally ISE (2011) Mango, Improving the taste of the tropics. In '1st Austropex, Australia Tropical Expertise Conference. (Advanced Cairns: Cairns, Australia, 12 August 2011)

Bally ISE (2010) Horticulture and Science breeders' workshop, Sunshine Coast, 3-5 November 2010

Bally ISE (2011) Avanços na Pesquisa e Desenvolvimento da Industria de Manga | [Advances in research and development of mango industry]. *Revista Brasileira de Fruticultura* **33**(1), 57-63.

Dillon NL, Innes DJ, Bally ISE, Wright CL, Devitt LC, Dietzgen RG. (2013) EST-SSR marker resources for diversity analysis of mango (*Mangifera indica* L.). *Diversity* (submitted).

Dillon NL, Bally ISE, Wright CL, Hucks L, Innes DJ, Dietzgen RG (2013) Genetic diversity of the Australian National Mango Genebank. *Scientia Horticulturae* **150**(0), 213-226.

Dillon NL, Innes DJ, Bally ISE, Smyth HE, Wright CL, Grice K, Dietzgen R (2013) The Australian mango fruit genomics initiative. In 'X International Mango Symposium', 4 – 7 June 2013, Punta Cana, Dominican Republic.

Dillon N, Bally ISE, Hucks L, Wright CL, Innes DJ, Dietzgen RG (2013) Implementation of SSR markers in mango breeding in Australia. *Acta Horticulturae* **992**, 259-267

Grice, K. and Bally I. S. E. (2013) "Screening for tolerance to anthracnose in mango" at the 9<sup>th</sup> Australian Mango Conference, 14-17 May 2013, Cairns.

Anthony D, Bally ISE (2011) Mango resistance fighters maintain good taste. DEEDI, Brisbane.

### **Visitors**

Discussions on the progress of breeding technologies have been undertaken with a series of visiting scientists and industry leaders who visited the project:

22/08/2003 – Dr Alok Kumar HAL, and Dr A. K. Singh, Indian Agricultural Research Institute

16/05/2013 - Dr Alok Kumar, HAL

22/04/2013 - Dr Jenny Margetts, Plant and Food

12/12/2012 – Dr Chitose Honsho, Miyazaki University, Japan and Dr Shinya Kanzaki, Kinki University, Japan

12/09/2012 – Professor Keizo Yonemori, Kyoto University, Japan

14/08/2012 – Mr Gavin Scurr, Chair AMIA, Mr John Nusifora, AMIA Board Member, and Trevor Dunmall, Industry Development Manager, AMIA.

03/03/2012 - Mr Khalid Mahmood, Agricultural department, Punjab, Pakistan, Prof. Nazim Labar, Bahauddin Zakariya University, Multan, Pakistan, Mr Hadi Leghari, Asim Agricultural Farm, TandoAlaya, Pakistan.

07/03/2012 - Dr Katja Kehlenbeck, ICRAF, Nairobi, Kenya

11/12/2011 - Indonesian visiting scientists

12/09/2011 - Dr Ray Schnell, USDA, Miami Florida

## Recommendations

As this project was about developing technologies to improve the efficiency of mango breeding in Australia, the recommendations concern the appropriateness of technology adoption into the Australian Mango Breeding Program and where further research is needed.

A key component of the project was to increase breeding capacity and efficiency by developing open pollination systems that use marker assisted selection to determine paternity of progeny. The project successfully showed that paternity of open pollinated mango progeny could be determined with molecular markers. In its current development this technology is somewhat restricted by parental pedigree, synchronisation of flowering and the efficiency of the SSR marker system visualised with polyacrylamide gel electrophoresis (PAGE). We recommend that open pollination and SSR-PAGE based marker assisted paternity testing be used where parents and progeny have distinctly distant pedigrees and adjacent trees have synchronous flowering. This recommendation may well change in the future as SSR markers are expected to be replaced with SNP markers that are discovered in the follow-on project to this "Intergrating genomics in to an applied mango breeding program (HAL MG13002). SNP markers will be easier and cheaper to run using contracted services and will be easy to run multiple markers for multiple traits, improving their efficiency.

The development of a recombination linkage map for mangoes was a goal of this project that was delayed due to -80°C freezer failures and changes of staff and projects in the USA. However, progress towards the development of the mango recombination map has been made and is continuing. We recommend that this work continues as it will be the source of many SNP markers and a platform on which to base further gene discovery and functionality research in mango.

The identification of new sources of postharvest disease resistance and rapid tests for their presence are exciting developments in the management of postharvest disease in mango. This project has progressed the science of identifying resistance to anthracnose disease in mango. Further discoveries of resistance and tolerance would be welcome as would a better understanding of disease resistance. We recommend that screening of varieties and genebank accessions for their tolerance to postharvest anthracnose disease continues. We also recommend that the work to develop a leaf assay for anthracnose resistance continues to explore new techniques, as the successful development of this test will significantly improve mango breeding efficiency where disease resistance is the target.

The authors would also like to see an ordinal scale of tolerance to anthracnose developed from previous and future screening of fruit. This ordinal data would be most useful in future breeding and genomic investigations. With the effort invested in developing mango breeding progeny from the anthracnose tolerant accessions, it is essential that the progeny from this family is screened for anthracnose tolerance when the trees begin to fruit. This information is essential for future breeding and for the discovery of the disease resistance genes. A full understanding of the mechanism, and host/ pathogen physiology of the tolerance observed in *M. laurina* cv. Lombok is lacking. Further research in to these aspects of the tolerance may help in its management and the discovery of the genes controlling it.

In the past our understanding of mango genetics has been limited by the relatively short time controlled breeding programs have been operating and the highly heterozygous nature of the species. With the advent and application of molecular genetics in our current breeding program as successfully demonstrated in this project we are gaining useful insights to mango genetics and what is possible in a mango breeding program. However, rapid development of mango genetics and its unique nomenclature make it difficult to understand for people outside of the discipline. For this reason we recommend that mango breeders communicate regularly with the Queensland industry to improve their understanding of what is and what is not possible in a mango breeding program. Such communication will assist the Australian mango industry in developing realistic expectations of mango breeding programs.

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